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# Plant Cells

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# Advances in Biochemical Engineering/Biotechnology

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# Plant Cells

**Volume Editor: J.-J. Zhong**

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# Preface

Plants produce more than 30,000 types of chemicals, including pharmaceuticals, pigments and other fine chemicals, which is four times more than those obtained from microbes. Plant cell culture has been receiving great attention as an alternative for the production of valuable plant-derived secondary metabolites, since it has many advantages over whole plant cultivation. However, much more research is required to enhance the culture productivity and reduce the processing costs, which is the key to the commercialization of plant cell culture processes. The recent achievements in related biochemical engineering studies are reviewed in Chapter 1. The effect of gaseous compounds on plant cell behavior has been little studied, and Chapter 2 focuses on these gas concentration effects (including oxygen, carbon dioxide, ethylene and others, such as volatile hormones like methyl jasmonate) on secondary metabolite production by plant cell cultures. Two metabolites of current interest, i.e., the antimalarial artemisinin (known as "qing hao su" in China) that is produced by *Artemisia annua* (sweet wormwood) and taxanes used for anticancer therapy that are produced by species of *Taxus,* are taken as examples. Bioprocess integration is another hot topic in plant cell culture technology. Because most of the plant secondary metabolites are toxic to the cells at high concentrations during the culture, removal of the product *in situ* during the culture can lead to the enhanced productivity. Various integrated bioprocessing techniques are discussed in Chapter 3.

To improve the productivity of commercially important compounds in plants or plant cell cultures, or even to produce completely new compounds, metabolic engineering of plant secondary metabolite pathways has opened a new promising perspective. Different strategies used for the genetic modification are discussed in Chapter 4, including single-gene and multiple-gene approaches, as well as the use of regulatory genes for increasing productivity. These approaches are, among others, illustrated with work on the terpenoid indole alkaloid biosynthesis. With the development of genetic engineering of plant cells or organs, a lot of recombinant products can be obtained in cheap plant cell culture media. Production of these high- value products in plant cells is an economically viable alternative to other systems, particularly in cases where the protein must be biologically active. Chapter 5 reviews foreign protein production from genetically modified plant cells, and the implications for future development of this technology are also discussed.

Plant micropropagation is another important application of plant cell culture, which is an efficient method of propagating disease-free, genetically uniform

and massive amounts of plants *in vitro*. The prospect of micropropagation through somatic embryogenesis provides a valuable alternative to the traditional propagation system, and the micropropagation of elite hairy roots offers other attractive advantages in the large-scale production of artificial seeds. Large-scale production of somatic embryos and hairy roots in appropriate bioreactors is essential if micropropagation and artificial seed systems are to compete with natural seeds. Chapter 6 identifies the problems related to large-scale plant micropropagation via somatic embryogenesis and hairy roots, and the most recent developments in bioreactor design are summarized. Emphasis is given to immobilization technology and computer-aided image analysis employed in the mass micropropagation. As promising materials in plant cell cultures, hairy roots are recently shown to be responsive to physical stimuli such as exposure to light. However, physiological properties of hairy roots caused by environmental conditions have been hardly investigated in engineering aspects. In Chapter 7, the authors have developed the photomixotrophic and photoautotrophic hairy roots of pak-bung (water spinach) from the heterotrophic originals under light conditions. The physiological and morphological properties and growth kinetics of these hairy roots have been characterized. The relationships between growth potential of photoautotrophic hairy roots and energy acquired by photosynthesis in the cells are discussed in terms of maintenance energy.

I would like to thank Professor Thomas Scheper, the managing editor of this series, and Dr. Marion Hertel, chemistry editorial of Springer-Verlag, for their strong support. The excellent work and very pleasant cooperation of Mrs. Ulrike Kreusel, desk editor (chemistry) of Springer-Verlag, is greatly appreciated. I am also grateful to the supports from the Cheung Kong Scholars Program of the Ministry of Education of China, the National Natural Science Foundation of China, and the East China University of Science and Technology.

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Jian-Jiang Zhong

# Contents

# Biochemical Engineering of the Production of Plant-Specific Secondary Metabolites by Cell Suspension Cultures

Jian-Jiang Zhong

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Plant cell culture has recently received much attention as a useful technology for the production of valuable plant-derived secondary metabolites such as paclitaxel and ginseng saponin. The numerous problems that yet bewilder the optimization and scale-up of this process have not been over emphasized. In spite of the great progress recorded in recent years towards the selection, design and optimization of bioreactor hardware, manipulation of environmental factors such as medium components, light irradiation, shear stress and $O_2$ supply needs detailed investigations for each case. Recent advances in plant cell processes, including high-density suspension cultivation, continuous culture, process monitoring, modeling and control and scale-up, are also reviewed in this chapter. Further developments in bioreactor cultivation processes and in metabolic engineering of plant cells for metabolite production are expected in the near future.

**Keywords.** Plant cell suspension culture, Secondary metabolite production, Bioprocess engineering, Bioreactor optimization, Environmental factors, Medium manipulation, Shear stress, Modeling monitoring and control, High density cell culture, Bioprocess scale-up, Metabolic engineering



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## 1 Introduction

The history of plant cell culture dates back to the beginning of this century, and since the 1930s a great deal of progress has been achieved. The concept of culturing plant cells includes the culture of plant organs, tissue, cells, protoplast, embryos, and plantlets. The application of plant cell culture has three main aspects: the production of secondary metabolites, micropropagation, and the study of plant cell genetics, physiology, biochemistry and pathology. This chapter reviews the recent advances in the optimization of bioreactor configurations and environmental factors for metabolite production by plant cell suspension culture, new developments in plant cell bioprocesses in bioreactors, and emerging research on metabolic engineering of plant cells.

## 2 Application of Plant Cell Culture to Production of Secondary Metabolites

Plant cell culture has several advantages as a method of producing useful plant-specific metabolites. Plants produce more than 30,000 types of chemicals, including pharmaceuticals, pigments and other fine chemicals, which is four times more than those obtained from microbes. Some of these chemicals are difficult to synthesize chemically, or it is difficult to produce or to increase the amounts produced by microorganisms through genetic engineering. Plant cell culture is not limited by environmental, ecological or climatic conditions and cells can thus proliferate at higher growth rates than whole plants in cultivation.

As shown in Table 1, some metabolites in plant cell cultures have been reported to be accumulated with a higher titer compared with those in the parent plants, suggesting that the production of plant-specific metabolites by plant cell culture instead of whole plant cultivation possesses definite merits and poten-

**Table 1.** Product yield from plant cell cultures compared with the parent plants. *DW* dry weight

| Product | Plant | Yield (% DW) | | Yield ratio (culture/ plant) |
|---|---|---|---|---|
| | | Culture | Plant | |
| Anthocyanin | *Vitis* sp. | 16 | 10 | 1.6 |
| | *Euphorbia milli* | 4 | 0.3 | 13.3 |
| | *Perilla frutescens* | 24 | 1.5 | 16 |
| Anthraquinone | *Morinda citrifolia* | 18 | 2.2 | 8 |
| Berberine | *Coptis japonica* | 13 | 4 | 3.3 |
| | *Thalictrum minor* | 10 | 0.01 | 1000 |
| Rosmarinic acid | *Coleus blumei* | 27.0 | 3.0 | 9 |
| Shikonin | *Lithospermum erythrorhizon* | 14 | 1.5 | 9.3 |

tial. Because plant cell culture is usually of low productivity, it can only be economically viable in the production of high-value added metabolites [1].

Generally, one main problem in the application of plant cell culture technology to secondary metabolite production is a lack of basic knowledge concerning biosynthetic routes and the mechanisms regulating the metabolite accumulation. Recently, there have been some reports addressing this important issue in plant cell cultures through elicitation, cell line modification by traditional and genetic engineering approaches, as well as biochemical study.

Elicitation is effective in enhancing metabolite synthesis in some cases, such as in production of paclitaxel by *Taxus* cell suspension cultures [2] and tropane alkaloid production by suspension cultures of *Datura stramonium* [3]. Increasing the activity of metabolic pathways by elicitation, in conjunction with end-product removal and accumulation in an extractive phase, has proven to be a very successful strategy for increasing metabolite productivity [4]. For example, two-phase operation with elicitation-enhanced alkaloid production in cell suspension cultures of *Escherichia californica* [5, 6].

Cell line selection is one of the traditional and effective approaches to enhancing metabolite accumulation, and biochemical studies provide the fundamental information for the intentional regulation of secondary metabolism in plant cells. In a carrot suspension culture regulated by 2,4-dichlorophenoxyacetic acid, Ozeki et al. [7] found that there was a correlation between anthocyanin synthesis and morphological differentiation for somatic embryogenesis; they also demonstrated the induction and repression of phenylalanine ammonia lyase (PAL) and chalcone synthase correlated with formation of the respective mRNAs. Two biosynthetic enzymes, i. e., PAL and 3-hydroxymethylglutaryl-CoA reductase, were also related with shikonin formation in *Lithospermum erythrorhizon* cultures [8].

Although plant cell culture has been demonstrated to be a useful method for the production of valuable secondary metabolites, many problems arise during bioprocess scale-up (Table 2). At present, there are only a few industrial processes in operation using plant cell cultures, which include shikonin, ginseng

**Table 2.** Problems in plant cell cultures

| Biological | Operational |
|---|---|
| Slow growth rate | Wall adhesion |
| Physiological heterogeneity | Light requirement |
| Genetic instability | Mixing |
| Low metabolite content | Shear sensitivity |
| Product secretion | Aseptic condition |

and paclitaxel production. Whether or not more products produced in this way will reach the market is strongly contingent on the economics of the process, which in turn is heavily dependent on the productivity of the culture. As mentioned above, selection of cell lines with suitable genetic, biochemical and physiological characteristics is an important point. Optimization of bioreactor configurations and environmental conditions is also definitely necessary to realize commercial production of useful metabolites by plant cells.

In the following sections, the environmental factors emphasized are medium components, light, shear, $O_2$, and rheology; the effect of gas concentration on secondary metabolite production by plant cell cultures has been reviewed by Linden et al. [9]. The developments in plant cell bioprocesses including high-density cell cultivations, continuous culture, process monitoring, modeling and control and process scale-up are focused; process integration for plant cell cultures has been reviewed by Choi et al. [10].

## 3 Design and Optimization of Bioreactor Hardware

Most of the bioreactors used to grow plant cells are usually directly derived from microbial fermenters. The choice and design of the most suitable reactor is determined by many factors, such as shear environment, $O_2$ transfer capacity, mixing mechanism, foaming problem [11], capital investment [12] and the need for aseptic conditions, related to the type of plant cells used and the purpose of the experiment [1]. Understanding how to promote better cell culture through reactor modification, e.g., impeller designs that produce reduced shear, and the efficient introduction of light, is a major challenge [13].

Until now, bioreactors of various types have been developed. These include loop-fluidized bed [14], spin filter, continuously stirred turbine, hollow fiber, stirred tank, airlift, rotating drum, and photo bioreactors [1]. Bioreactor modifications include the substitution of a marine impeller in place of a flat-bladed turbine, and the use of a single, large, flat paddle or blade, and a newly designed membrane stirrer for bubble-free aeration [13, 15–18]. Kim et al. [19] developed a hybrid reactor with a cell-lift impeller and a sintered stainless steel sparger for *Thalictrum rugosum* cell cultures, and cell densities of up to 31 g $l^{-1}$ were obtained by perfusion without any problems with mixing or loss of cell viability; the specific berberine productivity was comparable to that in shake flasks. Su and Humphrey [20] conducted a perfusion cultivation in a stirred tank bio-

reactor integrated with an internal cross-flow filter which provided $O_2$ without bubbles; a cell density of 26 g dry wt $l^{-1}$ and a rosmarinic acid productivity of 94 mg $l^{-1}$ $d^{-1}$ were achieved. In *Catharanthus roseus* cell cultures, a double helical-ribbon impeller reactor with a working volume of 11 l was successfully developed for high-density cultivation [21]. Yokoi et al. [22] also developed a new type of stirred reactor, called a Maxblend fermentor, for high-density cultivation of plant cells, and they demonstrated its usefulness in cultivations of rice and shear-sensitive *Catharanthus roseus* cells. In suspension cell cultures of *Perilla frutescens* for anthocyanin pigment production, an agitated bioreactor with a modified sparger and with internal light irradiation was developed, and the red pigment accumulation in the bioreactor was greatly enhanced compared with that obtained in a conventional reactor [23].

Recently, based on the principles of a centrifugal pump, a new centrifugal impeller bioreactor (CIB) (Fig. 1) has been designed for shear-sensitive biological systems by installing a centrifugal-pump-like impeller in a conventional stirred vessel [24, 25]. The fluid circulation, mixing, and liquid velocity profiles in the new reactor (5-l) were assessed as functions of the principal impeller design and bioreactor operating parameters. The performances of the CIB were compared with those of a widely used cell-lift bioreactor. The effects of the impeller configuration, aeration rate, and agitation speed on the oxygen transfer coefficient were also studied in the CIB. It was understood that the CIB showed higher liquid lift capacity, shorter mixing time, lower shear stress, and favorable oxygen transfer rate compared with the cell-lift counterpart. The

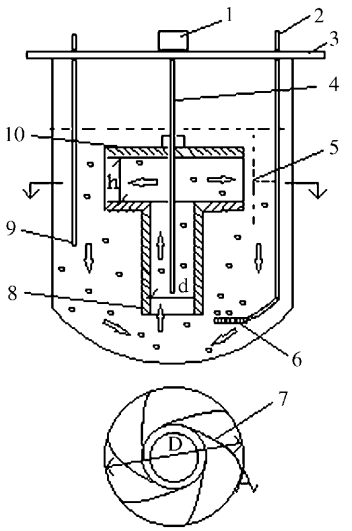


**Fig. 1.** Schematic diagram of a centrifugal impeller bioreactor (5 l). *1* Magnetic stirring device; *2* gas in; *3* head plate; *4* agitator shaft; *5* measurements of the liquid velocity profiles of the discharge flow were performed in the vertical direction across the width of the blades (*the vertical dashed line*) and at various radial distances from the impeller tip; *6* sintered stainless sparger; *7* centrifugal blade; *8* draft tube; *9* DO probe; *10* centrifugal rotating pan

CIB could have a promising future in its application in shear sensitive cell cultures [26].

Furthermore, the CIB was applied to cell suspension cultures of *Taxus chinensis* [27] and *Panax notoginseng* [28]. It was shown that, although the specific growth rate of *Taxus chinensis* cells in both the CIB and the cell-lift bioreactor was almost the same, the cells cultured in the CIB had shorter lag phase and less cell adhesion to the reactor wall compared with that in the cell-lift reactor [27]. The accumulation of anticancer diterpene paclitaxel was also higher in the CIB (data not shown). For high-density suspension cultivation of *Panax notoginseng* cells [28], the maximum cell density reached 28.9, 26.0 and 22.7 g $l^{-1}$ (by dry weight) in a shake flask (SF), the CIB and a turbine reactor (TR), respectively; their corresponding biomass productivity was 1103, 900 and 822 mg $l^{-1}$ $d^{-1}$. The total production of ginseng saponin reached about 0.92, 0.80, and 0.49 g $l^{-1}$ in the SF, CIB and TR, respectively; their corresponding saponin productivity was 34, 29 and 21 mg $l^{-1}$ $d^{-1}$. It can be concluded that, from the viewpoint of biomass production and saponin accumulation, the CIB was better than the TR, and the flask culture results can be well reproduced in the CIB.

Here, it can be seen that during the last decade research on the design of plant cell bioreactors has witnessed a boom and reached maturity. A future trend in this area is to combine bioreactor type with operational conditions for specific cell lines of characteristic morphology, physiology and metabolism, in order to optimize the processes for secondary metabolite production.

## 4
## Manipulation of Culture Environments

### 4.1
### Medium Manipulation

Plant cell culture medium includes phytohormone, inorganic and organic components. The effects of the medium employed on various processes have been reported, e.g., plant hormone effect in suspension cultures of *Panax quinquefolium* [29], effects of calcium and phosphate in the cultivation of *Coffea arabica* suspended cells [30], phosphate effects on saponin production in suspension cultures of *Panax ginseng, Panax notoginseng*, and *Panax quinquefolium* [31, 32], and the role of glucose in ajmalicine production by *Catharanthus roseus* cell cultures [33]. It is evident that medium manipulation is a very powerful way of enhancing the culture efficiency of plant cell cultures. A few more detailed examples, mainly from our laboratory regarding sucrose, nitrogen, potassium ion and medium feed, are illustrated below.

#### 4.1.1
#### *Sucrose*

Carbon source was found to be a significant factor in plant cell metabolism [34–39], which affected the accumulation of alkaloids by suspension cultures of *Holarrhena antidysenterica* [36], of anthocyanins by *Vitis vinifera* cell suspen-

sions [37], and of shikonin by *Lithospermum erythrorhizon* cell cultures [38]. A relatively higher concentration of sucrose was reported to be favorable to the rosmarinic acid production [34, 35].

In suspension cultures of ginseng cells (*Panax* spp.) for simultaneous production of ginseng saponin and ginseng polysaccharide, which both possess antitumor and immunological activities, manipulation of medium sucrose was demonstrated to be very effective for improvement of culture productivity [39]. The effect of initial sucrose concentration (i.e., 20, 30, 40 and 60 g $l^{-1}$) was investigated in cell cultures of *Panax notoginseng*. The final dry cell weight was increased with an increase in initial sucrose concentration from 20 to 40 g $l^{-1}$, but an even higher sucrose concentration of 60 g $l^{-1}$ seemed to repress the cell growth. A high sugar level was favorable to the synthesis of ginseng saponin, which may be due to the high osmotic pressure and reduced nutrient uptake (especially nitrate) under the conditions [39]. The content of ginseng polysaccharide was not apparently affected by initial sucrose levels. The maximum production of ginseng saponin and polysaccharide was achieved at an initial sucrose concentration of 40 g $l^{-1}$.

### 4.1.2 *Nitrogen*

Nitrogen source is also very important for plant cell metabolite formation, as reported in suspension cultures of *Holarrhena antidysenterica* for accumulation of alkaloids [36], in cell suspensions of *Vitis vinifera* for anthocyanin formation [37], and in shikonin production by *Lithospermum erythrorhizon* cell cultures [38].

The effects of nitrogen (N) source on the kinetics of cell growth, major nutrient consumption, and production of ginseng saponin and polysaccharide in suspension cultures of *Panax ginseng* cells have been studied [40]. The ratio of $NO_3^-/NH_4^+$ and initial total medium N were varied. Cell growth was better at 60 mM N and higher ratios of $NO_3^-/NH_4^+$. With nitrate alone or a mixture of nitrate and ammonium (at a ratio of 2:1) provided as N source, 10 mM N was found to be a crucial point for the cell mass accumulation. Nitrate was a favorable N source for ginseng cell growth, and a cell growth rate of 0.11 per day and a dry cell density of 13 g $l^{-1}$ were obtained at 10 mM of nitrate (sole N source). The results also indicate that the polysaccharide content was not much affected by a variation of the N source, while its maximum production of 1.19 g $l^{-1}$ was achieved at $NO_3^-/NH_4^+$ of 60:0 under 60 mM of total N (i.e., sole nitrate). The saponin production was relatively higher with an initial N concentration of 5–20 mM (with nitrate alone or at an $NO_3^-/NH_4^+$ ratio of 2:1).

### 4.1.3 *Potassium Ion*

Study of the potassium ion has been neglected [41] compared with other mineral ions like $Cu^{2+}$ [42, 43], although it plays important biochemical and biophysical roles in plant cells. $K^+$ serves as a major contributor to osmotic poten-

**Table 3.** Effects of initial $K^+$ concentration on the maximum content, production, productivity (Pv) and yield (against sugar) of ginseng saponin (*S*) and polysaccharide (*P*) in suspension cultures of *Panax ginseng* in shake flasks

| Initial $K^+$ (nM) | Content (mg $g^{-1}$) | | Production (g $l^{-1}$) | | Pv (mg $l^{-1}$ $d^{-1}$) | | Yield (%) | |
|---|---|---|---|---|---|---|---|---|
| | *S* | *P* | *S* | *P* | *S* | *P* | *S* | *P* |
| 0 | 50 | 100 | 0.35 | 0.72 | 6.5 | 14.2 | 1.0 | 2.1 |
| 5 | 54 | 95 | 0.44 | 0.78 | 7.3 | 15.8 | 1.3 | 2.3 |
| 10 | 54 | 98 | 0.45 | 0.81 | 7.6 | 16.6 | 1.3 | 2.7 |
| 20 | 48 | 91 | 0.45 | 0.88 | 7.6 | 18.6 | 1.4 | 2.8 |
| 30 | 63 | 90 | 0.62 | 0.89 | 14 | 19 | 1.9 | 2.5 |
| 40 | 71 | 91 | 0.70 | 0.90 | 14.6 | 23 | 2.3 | 3.0 |
| 60 | 76 | 90 | 0.73 | 0.84 | 15.4 | 21 | 2.2 | 2.8 |

tial, a specific requirement for protein synthesis, and an activator for particular enzyme systems. However, almost all previous studies have concentrated on cultivated plants, and there is only one early report related to $K^+$ effect on plant suspension cells [44]. In addition, it should be noted that during an investigation on the effects of nitrogen sources in plant cell cultures, in reality the $K^+$ amount is significantly altered because $KNO_3$ is a major medium component in conventional media. Thus, in such a case, the $K^+$ effect should also be clarified.

Table 3 shows the effects of initial $K^+$ concentrations within the range of 0–60 mM on the kinetics of cell growth, nutrient metabolism, and production of ginseng saponin and polysaccharide in suspension cultures of *Panax ginseng* cells, which were studied by varying $KNO_3$ and $NaNO_3$ concentrations at 60 mM of total nitrate (as the sole nitrogen source) [41]. It is understood that a higher $K^+$ concentration sustained a longer cell growth stage, and the changes in specific oxygen uptake rate (SOUR) of the cultured cells corresponded to the cell growth pattern with the maximum SOUR occurring before the growth peak. More soluble sugar was stored within the cells under $K^+$ deficiency. A curvilinear relationship between initial $K^+$ concentration and the active biomass accumulation (which was the total cell mass minus intracellular soluble sugars) was found, and the critical $K^+$ concentration was determined to be 20 mM. Furthermore, the results indicate that $K^+$ had little effect on the specific production (i.e., content) of ginseng polysaccharide; however, with an increase of initial $K^+$ within 0–20 mM, the total production of polysaccharide was increased gradually due to the increase in cell mass, and it leveled off at $K^+$ over 20 mM. On the other hand, the specific saponin production was remarkably enhanced with an increase of initial $K^+$ within 20–60 mM, and the maximum saponin production was achieved at an initial $K^+$ concentration of 60 mM.

#### 4.1.4 *Feeding*

Medium feeding is an effective way to improve the plant cell density and production of secondary metabolites [45–47]. For example, Wang et al. [46] de-

**Table 4.** Effects of feeding of carbon and nitrogen sources on cell growth and the content (C), production (Pn) and productivity (Pv) of ginseng saponin and polysaccharide. The cell culture in a modified MS medium was taken as a control. Cases 1, 2 and 3: Sucrose was fed beginning from day 15, 18 and 20, while its level was kept below 20, 10 and 20 g $l^{-1}$, respectively. Cases 4, 5 and 6: Feeding of 20 mM nitrate, 10 mM nitrate plus 10 mM ammonium, and 20 mM ammonium on day 15, respectively, where sugar addition was the same as in case 1

| Case | Dry cells (g $l^{-1}$) | Saponin | | | Polysaccharide | | |
|---|---|---|---|---|---|---|---|
| | | C (mg $g^{-1}$) | Pn (g $l^{-1}$) | Pv (g $l^{-1}$ $d^{-1}$) | C (mg $g^{-1}$) | Pn (g $l^{-1}$) | Pv (g $l^{-1}$ $d^{-1}$) |
| Control | 24.1 | 56.8 | 1.37 | 0.061 | 14.2 | 3.42 | 0.152 |
| 1 | 35.1 | 44.8 | 1.57 | 0.055 | 14.9 | 5.22 | 0.186 |
| 2 | 30.6 | 36.1 | 1.10 | 0.037 | 14.7 | 4.50 | 0.158 |
| 3 | 31.3 | 33.6 | 1.05 | 0.035 | 14.6 | 4.57 | 0.161 |
| 4 | 28.9 | 47.1 | 1.36 | 0.047 | 13.2 | 3.81 | 0.132 |
| 5 | 30.3 | 29.0 | 0.88 | 0.028 | 11.4 | 3.44 | 0.118 |
| 6 | 26.9 | 22.7 | 0.61 | 0.018 | 10.2 | 2.74 | 0.091 |

monstrated a conspicuous increase in taxane diterpene production by sucrose feeding. As shown by Fett-Neto et al. [47], paclitaxel yield was enhanced by aromatic carboxylic acid and amino acid feeding to cell cultures of *Taxus cuspidata*. Table 4 shows the effects of carbon and nitrogen source feeding on the cell cultures of *Panax notoginseng* [45]. In case 1, the highest dry cell weight of 35.1 g $l^{-1}$ was obtained, and, as observed, the cell sedimentation volume reached nearly 100%. The cell density reached may be the upper limit of this cell line. The highest production of ginseng saponin and polysaccharide was 1.57 and 5.22 g $l^{-1}$, respectively. The productivities of ginseng saponin and polysaccharide obtained were 2.8- and 3.4-fold higher than those reached in a conventional batch culture (i.e., in standard MS medium), respectively. The saponin content in case 1 was higher than that in case 2 (at a relatively low sugar level); this may be due to the fact that a relatively higher sugar concentration was favorable to ginseng saponin synthesis. Case 3 (with too late feeding) was also lower in saponin formation compared with case 1, which was probably due to the decline in cell activity. In the cases of 4, 5 and 6, feeding of nitrogen source during the later part of cultivation seemed to have a small negative effect on the cell growth, although sugar was fed at the same time as in case 1. This means that the initial nitrogen amount was enough for the high-density cell culture and the phenomenon of substrate inhibition may have occurred during cultivation. Based on the above results, the best approach for high-density cultivation of *Panax notoginseng* cells is to start the sugar feeding at the later exponential phase (i.e., day 15), and to keep the medium level below 20 g $l^{-1}$ by intermittent addition. This strategy has also been successfully applied to bioreactor cultivation of *Panax notoginseng* cells.

## 4.2 Light Irradiation

The spectral quality, intensity and period of light irradiation may affect plant cell cultures in one aspect or another [48]. Various research groups have demonstrated the stimulatory effect of light irradiation on the formation of compounds such as anthocyanins, vindoline, catharanthine, and caffeine in cell suspension cultures [48, 49]. Zhong et al. [48] investigated the quantitative effect of light irradiation intensity on anthocyanin formation by *Perilla frutescens* cell cultures, and found that the control of light intensity at 27.2 W/cm$^2$ was favorable to the red pigment production in a bioreactor.

## 4.3 Shear Stress

In suspension culture systems, the cells are subjected to hydrodynamic forces arising from mechanical agitation and sparging aeration. The effect of shear on biological cells has been investigated in various studies. Plant cells are perhaps not as sensitive as animal cells to hydrodynamic forces but are in general more sensitive than microbial cells because they have a large volume (with a size even larger than animal cells) and rigid (inflexible) cell wall. They also tend to form even larger aggregates up to a few mm in diameter [50, 51]. The cell morphology and aggregate size are very dependent on the hydrodynamic shear forces in the surrounding fluid. Therefore, plant cells are also considered to be shear-sensitive. In addition, plant cells in suspension culture tend to adhere to the walls of the culture vessels and accumulate at the headspace. At high cell densities, the biomass volume can take up 50–90% of the culture volume because of the high water content of plant cells. All these characteristics add complexity to operating large-scale bioreactors for suspension culture of plant cells [50, 51].

The shear-sensitivity of plant cells has been observed in a number of cases [17, 52–58]. For example, Takeda et al. [58] reported decreases in respiration rate, ATP content and membrane integrity of both eucalyptus and safflower cells under hydrodynamic stress in a stirred-tank reactor.

### 4.3.1 *Methods for Evaluating the Shear Effects on Plant Cells*

Quantitative investigations of shear effects on plant cells have been carried out in well-defined flow and shear fields using special flow and shearing devices such as Couette-type apparatus, recirculating flow capillary, submerged jet, or in well-characterized bioreactors. To date various methods have been developed and employed for the assessment of shear-related effects in plant cell suspension cultures (Table 5) [59, 60]. The evaluation of the shear effects on plant cells can be carried out by two approaches: a short-term shearing experiment to observe such parameters as viability and cell lysis, or a long-term bioreactor cultivation to determine the cell growth rate and metabolite productivity. Both cases are demonstrated below by examining our work on *Perilla frutescens* cells.

**Table 5.** Methods for assessing shear-related effects in plant cell suspension cultures [59, 60]. *TTC* Triphenyltetrazolium chloride

| System response | Parameter measured |
|---|---|
| Reduction in viability | Growth rate<br>Regrowth potential<br>Membrane integrity<br>Dye exclusion<br>Dual isotope labelling<br>Dielectric permitivity |
| Release of intracellular components | pH variation<br>Protein release<br>Total organic carbon<br>Secondary metabolite release |
| Change in metabolism | Oxygen uptake rate<br>Respiration activity (TTC reduction)<br>ATP concentration<br>Metabolite productivity<br>Cell wall composition |
| Changes in morphology and/or aggregation patterns | Aggregate size/shape |
| Growth and lysis kinetics | Expansion index<br>Esterase release |

### 4.3.2 *Effects of Shear Stress on Plant Cells in a Bioreactor*

Plant cells cultivated in a bioreactor may be affected by shear stress created by agitation and/or aeration during a long-term exposure to even a low-shear environment. Here, a quantitative investigation of the influence of hydrodynamic shear on the cell growth and anthocyanin pigment production is demonstrated by using a plant cell bioreactor with marine impellers of larger (85 mm) and smaller (65 mm) diameter (Fig. 2) [61]. Figures 2 A and 2 B show the effect of the average and maximum (represented by the impeller tip speed) shear rates on the specific growth rate of *Perilla frutescens* cells in a bioreactor. The specific growth rate was apparently reduced at an average shear rate over 30 $s^{-1}$ or at an impeller tip speed of over 8 dm $s^{-1}$. Figure 2 C and 2 D show the influence of shear on the maximum cell concentration of the cell cultures. The maximum cell concentration was relatively higher at an average shear rate of 20 – 30 $s^{-1}$ or an impeller tip speed of 5 – 8 dm $s^{-1}$. The cell density was reduced at an average shear rate over 30 $s^{-1}$ or at an impeller tip speed over 8 dm $s^{-1}$.

The effect of shear stress on the pigment content, i. e., its specific production, of the *Perilla frutescens* cells in the reactor is shown in Figs. 3 A and 3 B. The anthocyanin content was relatively high at an average shear rate below 30 $s^{-1}$ or an impeller tip speed below 8 dm $s^{-1}$. At higher shear rates, the pigment content of the cultured cells showed an obvious decrease. The effects of shear on the total production and the volumetric productivity of anthocyanin in the bioreactor were also similar (data not shown). The pigment production and pro-

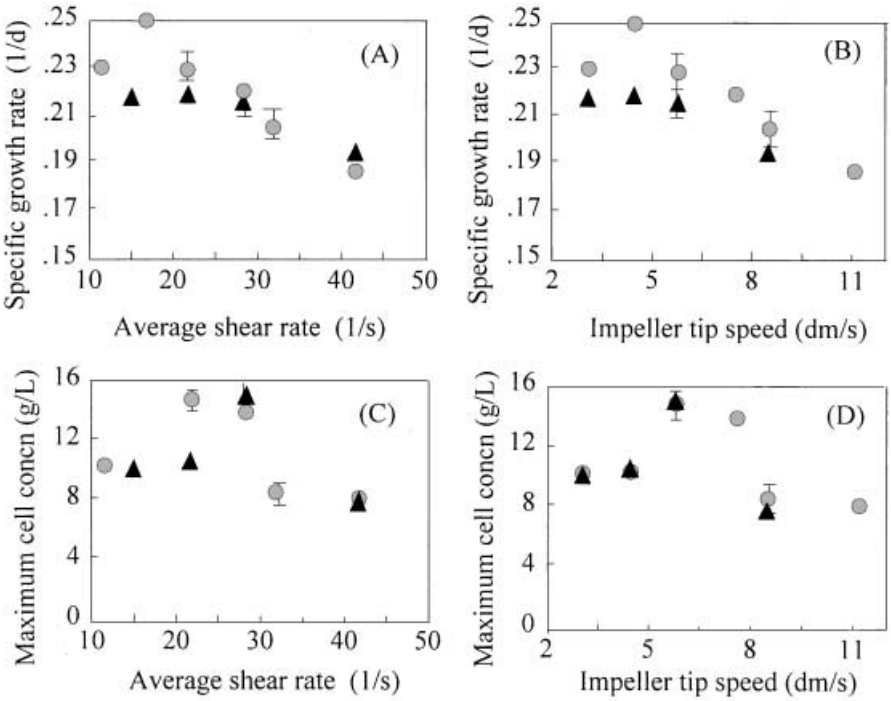


**Fig. 2.** Influences of shear on specific growth rate and maximum cell concentration of *Perilla frutescens* cells in a plant cell reactor with a marine impeller. *Circles* larger impeller (85 mm diam.); *triangles* smaller impeller (65 mm diam.)

ductivity were relatively higher at an average shear rate between 20–30 $s^{-1}$ or an impeller tip speed between 5–8 dm $s^{-1}$, and they decreased at higher shear rates. The effect of shear rate on the yield coefficient of anthocyanin against sucrose in the cell cultures is shown in Figs. 3C and 3D. A relatively higher product yield was observed at an average shear rate between 20–30 $s^{-1}$ or an impeller tip speed between 5–8 dm $s^{-1}$.

Such information about shear effects in plant cell suspension cultures as given above is useful for bioreactor design and operation as well as the optimization of bioreactor environments for plant cell cultures. It may also be beneficial to bioreactor scale-up and high-density cultivation of plant cells for production of useful plant-specific chemicals (pharmaceuticals). In addition, because different cell suspensions can show different degrees of cell sensitivity to shear stress, and shear affects culture viability, cell lysis, and even metabolite secretion, as demonstrated in various cases like cell cultures of tobacco, *Catharanthus roseus* and *Perilla frutescens* [17, 54, 61], detailed studies are required for individual cases.

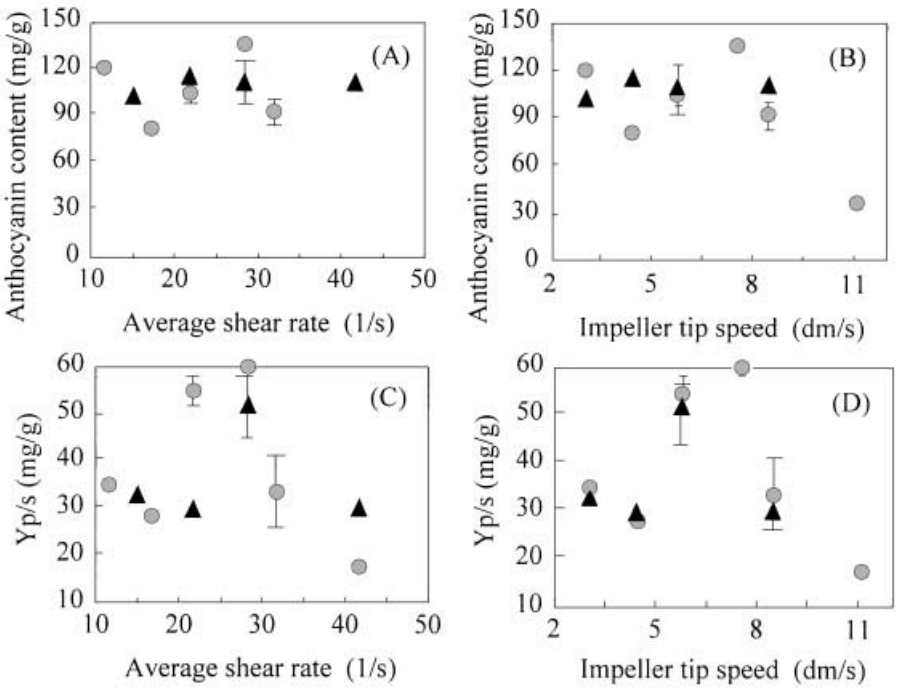


**Fig. 3.** Effects of shear on anthocyanin content and product yield in bioreactor cultures of *Perilla frutescens* cells. The symbols are the same as in Fig. 2

### 4.4 Oxygen Supply

$O_2$ supply affects both growth and metabolite production in a number of plant cell cultures including *Perilla frutescens* [23] and *Catharanthus roseus* [62]. In flask cultures of *Thalictrum minus* cells for berberine production, berberine-producing cells were observed to take up twice as much $O_2$ as nonproducing cells [63]. Gao and Lee [64] also demonstrated that an increase in the $O_2$ supply improved the specific $O_2$ uptake rate, the formation of a foreign protein ($\beta$-glucuronidase), and secondary metabolite (phenolics) production in flask and bioreactor cultivations of tobacco cells. Recently, Schlatmann et al. [65, 66] reported the effects of oxygen and dissolved oxygen concentration on ajmalicine production and related enzyme activities in high-density cultures of *Catharanthus roseus*.

For anthocyanin production by cell cultivation of *Perilla frutescens* in an agitated bioreactor, at an aeration rate of 0.1 vvm using a sintered sparger, its accumulation was poor, showing almost the same result as that at 0.2 vvm using a ring sparger, when the other cultivation conditions were maintained the same [23]. However, when the aeration rate was increased to 0.2 vvm with the sinter-

ed sparger being used, the product formation increased to almost threefold (i.e., 1.65 g $l^{-1}$). This result suggests that the oxygen supply conditions had a conspicuous effect on secondary metabolite production.

### 4.5 Rheology

The rheology of plant cell suspension cultures is important for the development and improvement of cell cultivation processes. Knowledge of the rheology of plant cell cultures may suggest solutions to various problems because culture viscosity, mixing, mass transfer, shear stress and cell growth, as well as metabolite production, all interact in bioreactor cultivation [67–70]. Cell cultures of *Perilla frutescens* were found to exhibit Bingham plastic fluid characteristics, and the size of the individual cells, not the cell aggregates, affected the rheological characteristics [67]. Ballica et al. [71] studied the rheological properties and determined the yield stress value of *Datura stramonium* cell suspensions. Curtis and Emery [72] demonstrated the rheological characteristics of 10 different plant cell suspension cultures, and claimed that most plant cell suspensions displayed Newtonian behavior at moderate cell densities, and non-Newtonian behavior was a result of cellular elongation observed in only a few cell lines.

With an increase in apparent viscosity of cell suspension, a significant decrease in $K_La$ was observed [73]. The results of oxygen supply effects on anthocyanin production by *Perilla frutescens* [23] imply that the efficiency of the production process may be reduced with an increase in the culture viscosity during cultivation. In addition, the problem of oxygen supply may become more serious in high-density bioreactor cultivations where poor mixing and heavy sedimentation can occur, thus improvement in metabolite production may become difficult. Recently, there has been an interesting report about the regulation of broth viscosity and $O_2$ transfer coefficient by osmotic pressure in cell suspensions of *Panax notoginseng* [74], while a relatively higher osmotic pressure was found favorable to the saponin accumulation by the cells [75].

## 5 Advances in Bioreactor Cultivation Processes

### 5.1 High-Density Cell Culture

Until now, the limited commercial application of plant cell cultures can be attributed to matters of production cost, while economic feasibility studies are unanimous in acknowledging productivity as a limiting factor. Due to the fact that productivity depends on product yield, the organism growth rate and prevailing biomass levels, high-density cell culture systems offer the advantage of production of metabolites in a compact cultivation vessel with high volumetric production rate (i.e., productivity), especially for intracellular products. For example, in *Perilla frutescens* cell cultures for anthocyanin production [76] and in

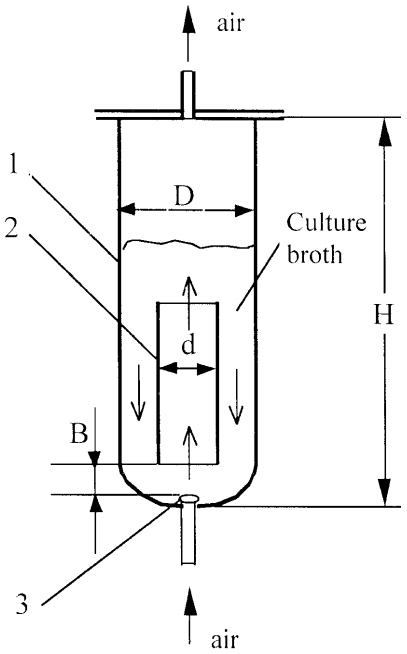


**Fig. 4.** Schematic diagram of a concentric-tube airlift bioreactor (with working volume of 1.0 l). *1* Reactor column; *2* inner draft tube (with its height of 15 cm); *3* sparger; *B* bottom clearance (2.5 cm); *d* diameter of draft tube (4.5 cm); *D* reactor diameter (7.0 cm); *H* column height (40 cm)

cultivation of *Panax notoginseng* cells in stirred bioreactors for the production of ginseng biomass and ginseng saponin [28], high-density cultivation has been successfully demonstrated.

A feeding strategy was studied in an airlift bioreactor (Fig. 4) in order to enhance the productivities of both ginseng saponin and ginseng polysaccharide on a reactor scale. It was found that sucrose should be fed before the specific oxygen uptake rate (SOUR) experiences a sharp decrease (at day 13) during the cultivation, i.e., the SOUR drop rate should not be larger than 0.5 mg $O_2$ (g DW)$^{-1}$ h$^{-1}$ d$^{-1}$. The added sugar could not be efficiently used for cell growth and metabolite synthesis if it was fed when the SOUR of the cells had already undergone a sharp drop and was very low (Table 6).

Figure 5 shows the kinetics of cell growth and sugar consumption in the airlift fed-batch cultivation (AFB). It indicates that sugar feeding did promote the final cell density, and a very high cell concentration of 29.7 g DW l$^{-1}$ was successfully achieved on day 17. A very high biomass productivity of 1518 mg DW l$^{-1}$ d$^{-1}$ was also obtained in this case. The cell density was comparable to a previous record in flask cultures [45], while the productivity obtained here was much higher than that case, where it was 1188 mg DW l$^{-1}$ d$^{-1}$ [75]. In high-density cultures of carrot cells in a 1-l airlift reactor, the highest cell density obtained was 15.2 g l$^{-1}$ ($1.09 \times 10^7$ cells ml$^{-1}$) [77]. In the case of *Catharanthus roseus*

**Table 6.** Comparison of production and productivity of cell mass (by DW), saponin and polysaccharide under different feeding strategies

| Feeding time | | Day 13 | Day 15 |
|---|---|---|---|
| SOUR (mg $O_2$ $g^{-1}$ (DW) $h^{-1}$) | | 7.9 ± 0.5 | 4.2 ± 0.6 |
| Total production ($g\ l^{-1}$) | Dry cells | 29.7 ± 1.3 (17)[a] | 26.7 ± 0.8 (17)[a] |
| | Saponin | 2.1 ± 0.2 | 1.7 ± 0.1 |
| | Polysaccharide | 3.0 ± 0.3 | 2.5 ± 0.2 |
| Productivity ($mg\ l^{-1} \cdot d^{-1}$) | Dry cells | 1518 ± 82 | 1347 ± 53 |
| | Saponin | 106 ± 14 | 88 ± 10 |
| | Polysaccharide | 158 ± 19 | 125 ± 14 |

[a] The number in parentheses means the time (day) when the maximum cell dry weight was obtained, and the maximum metabolites' production was also reached on the same day. The data are expressed as an average with standard deviation, which was calculated from three samples.

suspension cultures, the maximum biomass accumulation obtained in an airlift reactor was 23.4 g $l^{-1}$, although a higher cell density over 30 g $l^{-1}$ was achieved in a shake flask control [78].

A fluctuation in saponin content was seen in the AFB (Fig. 5B). The final production of ginseng saponin in the AFB reached about 2.1 g $l^{-1}$, and its productivity was 106 mg $l^{-1}$ $d^{-1}$. Both the production titer and volumetric productivity were much higher than those previously reported on a flask scale [45]. In the AFB, the dynamic change in polysaccharide content ranged from 7.3 mg (100 mg DW)$^{-1}$ to 10.2 mg (100 mg DW)$^{-1}$ (Fig. 5C). Generally, it increased with an increase in cell mass from day 5. Because of the high cell density, the total production of polysaccharide in the bioreactor was up to 3.03 g $l^{-1}$ on day 17, and its productivity reached 158 mg $l^{-1}$ $d^{-1}$.

### 5.2 Continuous Culture

Theoretically speaking, continuous culture is most promising for obtaining a high fermentation productivity. Seki et al. [79, 80] and Phisalaphong and Linden [81] demonstrated the enhancement of paclitaxel production by continuous cell culture of *Taxus cuspidata* and by using a semicontinuous culture of *Taxus canadensis* with total cell recycle, respectively. However, due to the practical problems of cell line stability and long-term sterile operation of bioreactors, such work is still limited in the laboratory. On the other hand, as shown by Van Gulik et al. [82], a chemostat culture technique is useful to obtain reliable data on the stoichiometry of the growth of plant cells in a bioreactor. Several other groups have also studied growth kinetics, stoichiometry, and modeling of the growth of suspension-cultured plant cells by using semicontinuous or fed-batch cultures to achieve steady-state growth. In addition, Westgate et al. [83] presented fed-batch cultivation kinetics for continuous approximation in *Cephalotaxus harringtonia* cultures.

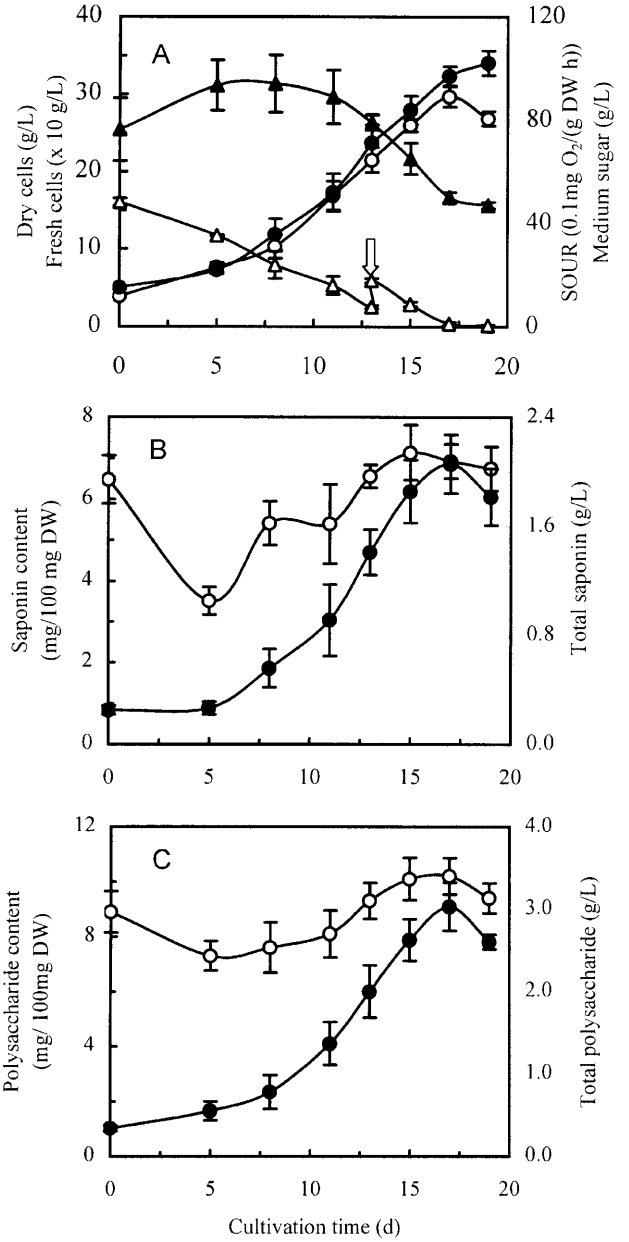


**Fig. 5.** Time profiles of **A** fresh and dry cell weights, medium sugar and SOUR, **B** the content and total production of ginseng saponin and **C** polysaccharide in fed-batch cultivation of *Panax notoginseng* cells in 1-l airlift reactors. The arrow in **A** indicates the sucrose feeding point. The error bars in the figure indicate the standard deviations for the samples of three identical reactors. Symbols: **A** *Dark circles* FW; *open circles* DW; *open triangles*, medium sugar; *dark triangles*, SOUR. **B** *Dark circles* Saponin production; *open circles* saponin content. **C** *Dark circles* Polysaccharide production; *open circles* polysaccharide content

## 5.3 Process Monitoring, Modeling and Control

For a better understanding of the dynamic changes in the physiological state of cells during cultivation, as well as for control and optimization of bioprocesses, monitoring of physiological variables is very important. For example, the shear damaging effects could be well understood by monitoring the $O_2$ uptake rates of suspended strawberry cells [84]. In spite of a pressing need for better monitoring and control in the optimization of plant cell bioprocesses, few studies have been published in this area. The monitoring parameters most frequently reported in plant cell cultures are the cell concentration, the NAD(P)H concentration [85], and the $O_2/CO_2$ concentrations in reactor inlet and outlet gases [86]. The monitoring of cell concentration is one of the most attractive areas of research [87], and in plant cell suspension culture it has been performed by conductometry [88, 89], osmotic pressure measurement [90], the dielectric measurement [91, 92] and turbidimetry [93, 94], and it can also be estimated by a neural-network approach [95].

Furthermore, in pigment production by cell suspension cultures, Smith et al. [96] demonstrated a nondestructive machine vision analysis of pigment-producing cells, and Miyanaga et al. [97] analyzed pigment accumulation heterogeneity using an image-processing system. Recently, Yanpaisan et al. [98] used flow cytometry to monitor cell cycle activity and population on dynamics in heterogeneous plant cell suspensions. Such work provides an opportunity for fine-tuning bioprocesses based on cell-level phenomena.

### 5.3.1 *In Situ Monitoring in Shake Flasks*

In plant cell cultures, shake flask culture is an indispensable stage of cultivation. Investigations in a shake flask are very essential and critical to bioprocess scale-up and optimization. We have developed a simple and convenient technique based on the principle of the Warburg manometric method to measure $O_2$ uptake rate (OUR) and $CO_2$ evolution rate (CER) of suspended cells in a shake flask culture. This technique has been successfully applied to suspension cultures of *Panax notoginseng* cells, and some important bioprocess parameters, such as OUR, CER, respiratory quotient (RQ), SOUR and specific CER (SCER), were quantitatively obtained [99]. As long as the environment temperature is strictly controlled to within an error of 0.1 °C, the measuring system is accurate and reproducible, is easy to operate, is economical, and is also able to treat many samples simultaneously.

### 5.3.2 *On-Line Monitoring of Bioreactor Processes*

A computer-aided on-line monitoring system was established and applied for plant cell bioprocesses, with *Perilla frutescens* as a model cell [86]. The cell concentration was successfully measured on-line and *in situ* with a laser turbidi-

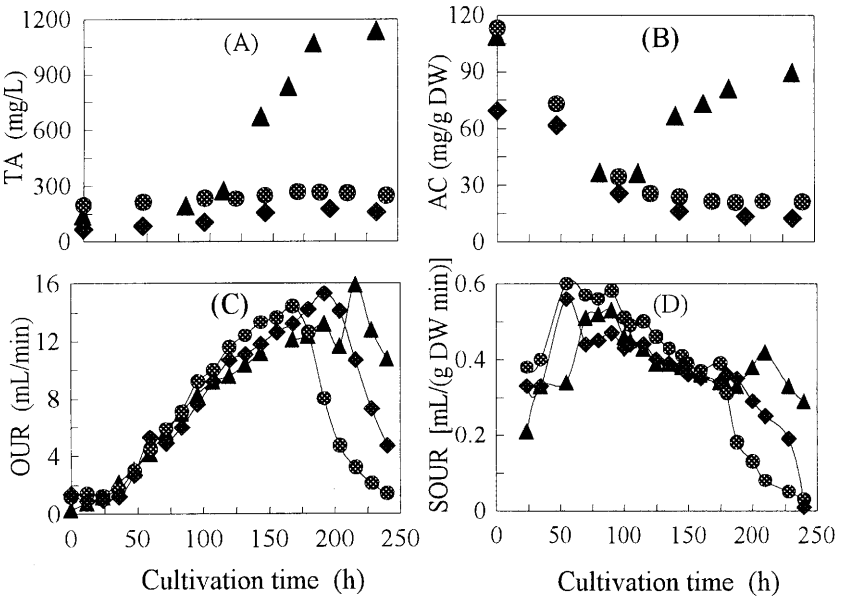


**Fig. 6.** Dynamic behaviors in total anthocyanin (TA), anthocyanin content (AC), oxygen uptake rate (OUR) and specific oxygen uptake rate (SOUR) in a bioreactor. *Triangles* 150 rpm; *circles* 300 rpm; *diamonds* 400 rpm agitation speed

meter (at 780 nm) [94]. Using this system, a detailed analysis of the response of the cells' physiological and metabolic aspects such as cell growth and respiratory activity under different agitation speeds was conducted. The system was useful for the selection of some informative process variables and for the identification of the physiological states of plant cells during bioreactor cultivations. The process variables, such as RQ, pH, and SCER, could be used for the identification of the growth phase of the cells during cultivation. The experimental results also suggest that the process variables, i.e., OUR and SOUR, may be related to the accumulation of anthocyanin in *Perilla frutescens* cell cultures (Fig. 6). Monitoring of the above variables including OUR and SOUR during a bioreactor cultivation may be very useful for effective feeding and control in a high-density cell culture to optimize the product accumulation. The information provided by this work could be helpful for the control and optimization of plant cell bioprocesses [86].

### 5.3.3
### *Mathematical Modeling and Process Control*

Mathematical models of biological processes are often used for hypothesis testing and process optimization. Using physical interpretation of results to obtain greater insight into process behavior is only possible when structured models that consider several parts of the system separately are employed. A number of dynamic mathematical models for cell growth and metabolite pro-

duction by plant cells have been developed for different systems by Bailey and Nicholson [100], Bramble et al. [30], Curtis et al. [101], Hooker and Lee [102], and Van Gulik et al. [103]. By taking account of culture interactions with pathways designated for structural component production, secondary metabolite synthesis and cellular respiration, a basic, structured kinetic model was successfully offered for application to batch suspension cultures of tobacco [102]. In suspension cultures of *Symphytum officinale*, polysaccharide production was investigated by experiments and mathematical modeling [104]. Schlatmann et al. [105] showed a simple, structured model for maintenance, biomass formation, and ajmalicine production by nondividing *Catharanthus roseus* cells.

Several interesting parameter-control models or systems have been reported in recent years. These are a five-state mathematical model for temperature control by Bailey and Nicholson [106], a mathematical-model description of the phenomenon of light absorption of *Coffea arabica* suspension cell cultures in a photo-culture vessel by Kurata and Furusaki [107], and a bioreactor control system for controlling dissolved concentrations of both $O_2$ and $CO_2$ simultaneously by Smith et al. [108].

Because bioprocesses are time-dependent and nonlinear in nature, the enormous complexity and uncertainty of plant cell processes require a sophisticated operational logic, which cannot easily fit into the mathematical framework of the traditional control approach. Here, a physiological-state control approach, in which the current physiological state of a cell culture is monitored [86], may be a powerful method for the control of plant cell processes, because, being based on artificial intelligence methods, in particular fuzzy sets and pattern recognition theory, no conventional mathematical model is required for the synthesis of such a control system. In reality, for the design and control of microbial processes, the introduction of such a knowledge-based methodology has proven useful when working with the fragmentary, uncertain, qualitative and blended knowledge typically available for the processes [109].

## 5.4 Bioprocess Scale-Up

During the scale-up of plant cell cultures, reduced productivity has often been reported [110, 111]. This reduction could be attributed to various factors such as shear stress, oxygen supply, and gas composition. As described above, plant cells are sensitive to shear stress, and oxygen supply is also significant in affecting secondary metabolite formation in plant cell cultures [23, 62, 64]. Gas exchange between the gas and liquid phases is another important factor that may affect the scale-up of plant cell cultures. In bioreactors, forced aeration is needed to supply oxygen and to improve fluid mixing. However, it may also lead to the removal of some known (such as $CO_2$ and ethylene) or unknown gaseous compounds. Such gaseous metabolites were proven or suggested to be important for cell growth and/or synthesis of secondary metabolites in plant cell and tissue cultures [9, 64, 112, 113]. In a shake flask there is no forced aeration and gaseous metabolites can be accumulated in its headspace. Consequently, the concentrations of the dissolved gases may be quite different between shake

flasks and bioreactors, and, for different culture systems, the effects of gaseous compounds may also be different. Schlatmann et al. [114] studied the relationship between the dissolved gaseous metabolites and the specific ajmalicine production rate in *Catharanthus roseus* cultures by air recirculation in a turbine reactor, and their results indicated that a higher concentration of dissolved gaseous metabolites reduced the ajmalicine production. However, in suspension cultures of *Thalictrum rugosum* in an airlift reactor, carbon dioxide and ethylene were shown to have stimulatory effects on alkaloid production [113]. Mirjalili and Linden [115] investigated the effects of different concentrations and combinations of oxygen, carbon dioxide and ethylene on the cell growth and paclitaxel production in suspension cultures of *Taxus cuspidata* on a flask scale. They found that the most effective gas mixture composition in terms of paclitaxel production was 10% (v/v) oxygen, 0.5% (v/v) carbon dioxide, and 5 ppm ethylene.

In suspension cultures of *Taxus chinensis*, it was noted that a considerable drop in taxuyunnanine C (TC, a novel bioactive compound) accumulation occurred when the cells were transferred from a shake flask to a bioreactor [116]. A systematic approach was taken by performing a series of experiments in combination with theoretical analyses to identify the potential factors that may be responsible for the process scale-up [117]. Finally, ethylene incorporation into the inlet air was carried out to determine whether the reduction of taxane production in bioreactors was due to the stripping-off of ethylene during cultivation. As shown in Table 7, it is clear that the ethylene had a promoting effect on the cell growth, and the maximum biomass reached 16.3 and 18.5 g $l^{-1}$ at an ethylene concentration of 6 and 18 ppm, respectively. The values were much higher than that of the control bubble column reactor, and even a little higher than the shake flasks. As to the accumulation of TC, its content was similar to the control bioreactor when 6 ppm of ethylene was incorporated into the inlet airflow, and it was obviously much less than that in the shake flasks. However, when 18 ppm of ethylene was directed into the bioreactor, the TC content was greatly enhanced and reached the same level as in the shake flasks at the end of cultivation (day 17). Therefore, although the effects of ethylene on secondary metabolism of plant cell cultures are dependent on the cell species and specific cell lines as used [113, 115, 118–120], the above example indicates that ethylene

**Table 7.** Cell growth and TC accumulation by *Taxus chinensis* cells in Erlenmeyer flasks and bubble column reactors incorporated with different levels of ethylene

| | Flask | Bubble column | | |
|---|---|---|---|---|
| Ethylene concentration (ppm) | 0 | 0 | 6 | 18 |
| Maximum biomass (g $l^{-1}$) | 14.6 | 11.7 | 16.3 | 18.5 |
| Average growth rate ($d^{-1}$) | 0.17 | 0.14 | 0.19 | 0.22 |
| Maximum TC content (mg $g^{-1}$ DW) | 13.3 | 6.2 | 7.0 | 14.2 |
| Maximum TC production (mg $l^{-1}$) | 178.8 | 70.7 | 111.4 | 229.0 |
| TC productivity (mg $l^{-1}$ $d^{-1}$) | 10.5 | 5.1 | 6.6 | 13.5 |

is a key factor in the scale-up of suspension cultures of *Taxus chinensis* from a shake flask to a laboratory bioreactor. The information obtained is very useful for a large-scale bioreactor cultivation of the plant cell.

## 6 Metabolic Engineering of Plant Cells for Metabolite Production

Metabolic engineering of cells is a natural outcome of recombinant DNA technology. In a broader sense, metabolic engineering can be viewed as the design of biochemical reaction networks to accomplish a certain objective. Typically, the objective is either to increase the rate of a desired product or to reduce the rate of undesired side- products, or to decompose the toxic or undesired substances. Of central importance to this field is the notion of cellular metabolism as a network. In other words, an enhanced perspective of metabolism and cellular function can be obtained by considering the participating reactions in their entirety, rather than on an individual basis [121]. This research field is, therefore, multidisciplinary, drawing on information and techniques from biochemistry, genetics, molecular biology, cell physiology, chemistry, chemical engineering, systems science, and computer science [122].

To improve the productivity of commercially important compounds in plants or plant cell cultures, or even to produce completely new compounds, metabolic engineering of plant secondary metabolite pathways has opened up a new promising perspective [123, 124]. In *Taxus chinensis* cell cultures, Srinivasan et al. [125] examined the dynamics of taxane production and metabolic pathway compartmentation by using metabolic inhibitors, elicitors, and precursors. Potential yield-limiting steps were predicted, which was useful in facilitating the metabolic engineering of *Taxus* cell cultures for paclitaxel production. Verpoorte et al. [126] introduced both the genes encoding tryptophan decarboxylase and strictosidine synthase from *Catharanthus roseus* into tobacco, and the cells were capable of synthesizing strictosidine after feeding of secologanin. For metabolic engineering of cultured tobacco cells, Shinmyo and co-workers [127] constructed a gene expression system by isolating three cDNA clones, which showed a high mRNA level in the stationary growth phase. They claimed that promoters of these genes would be useful in gene expression in high cell-density culture. In another aspect, as suggested in the case of Chinese hamster ovary cells by Fussenegger et al. [128], coordinated manipulation of multiple genes may also be required for the effective reprogramming of complex metabolism regulatory apparatus of plant cell cycle to achieve bioprocess goals, such as cessation of proliferation at high cell density to allow an extended period of high production.

In recent years, there have been many developments in biochemical and molecular-genetic studies in plants, such as biosynthetic pathways and metabolism of terpenoids [129] and cloning and expression of triterpene synthase cDNAs from triterpene-producing plants or plant cells [130]. Rapid progress in metabolic engineering of plant cells for highly efficient production of useful metabolites in cell cultures is expected in the near future. Also, by adopting metabolic engineering tools, the exploitation of the molecular diversity and con-

trol of the heterogeneity of plant cell metabolites such as ginsenosides may be possible, which will be very meaningful to the production and application of various medicinal plants including many famous traditional Chinese herbs [131].

## 7 Conclusions and Future Perspectives

Plant cell culture can produce many valuable metabolites including novel medicinal agents and recombinant products [132–136]. In combination with synthetic chemistry, the methodology also affords an attractive route to the synthesis of complex natural products and related compounds of industrial importance [137]. In recent years, the market for natural plant products has expanded rapidly, and this trend will continue because more and more people prefer to use natural products. Plant cell culture will contribute more to the market with the technological advancement in the future. For example, the current world market of raw materials of ginseng is about one billion US$ [138]; although cell-cultured ginseng occupies less than 1% of the market, its share will increase greatly with enhancement of the culture productivity.

In this chapter, it has been demonstrated how the significance of the design and optimization of bioreactor hardware, the manipulation of culture environments including medium (e.g., sucrose, $N$, $K^+$, feeding), light, shear, $O_2$ and rheology, as well as the development of new bioreactor cultivation processes such as high-density cultivation, continuous culture, process monitoring, modeling and control, and bioprocess scale-up, can enhance the productivity of plant cell suspension cultures for useful metabolite production. There is reason to believe that a significant advance in the process control and optimization of plant cell cultures will be achieved in the near future, although at present there are two main obstacles – the lack of an adequate process monitoring and control system for plant cells and the heterogeneity and instability of the cells. Furthermore, a breakthrough in the metabolic engineering of plant cells, such as considerable improvements in cell physiology and secondary metabolism, will ultimately enhance the productivity of many plant cell processes and push them to an economically feasible stage for commercial production of useful metabolites. In the future, an intimate cooperation between biologists and biochemical engineers is necessary that will require researchers in both these disciplines to expand their knowledge bases and research fields to the hybrid cutting-edge.

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## References


1. Zhong JJ, Yu JT, Yoshida T (1995) World J Microbiol Biotechnol 11:461
2. Yukimune Y, Tabata H, Higashi Y, Hara Y (1996) Nat Biotechnol 14:1129
3. Bellica R, Ryu DDY, Kado CI (1993) Biotechnol Bioeng 41:1075
4. Brodelius P, Pedersen H (1993) Trends Biotechnol 11:30
5. Byun SY, Ryu YW, Kim C, Pedersen H (1992) J Ferment Bioeng 73:380
6. Byun SY, Pedersen H (1994) Biotechnol Bioeng 44:14
7. Ozeki Y, Komamine A, Tanaka Y (1990) Physiol Plant 78:400
8. Srinivasan V, Ryu DDY (1992) Biotechnol Bioeng 40:69
9. Linden JC, Haigh JR, Mirjalili N, Phisaphalong M (2001) this volume
10. Choi JW, Cho GH, Byun SY, Kim DI (2001) this volume
11. Zhong JJ, Seki T, Kinoshita S, Yoshida T (1992) World J Microbiol Biotechnol 8:106
12. Hsiao TY, Bacani FT, Carvalho EB, Curtis WR (1999) Biotechnol Prog 15:114
13. Treat WJ, Engler CR, Soltes EJ (1989) Biotechnol Bioeng 34:1191
14. Dubuis B, Kut OM, Prenosil JE (1995) Plant Cell Tiss Org Cul 43:171
15. Boehme C, Schroeder MB, Jung-Heiliger H, Lehmann J (1997) Appl Microbiol Biotechnol 48:149
16. Hooker BS, Lee JM, An G (1990) Biotechnol Bioeng 35:296
17. Leckie F, Scragg AH, Cliffe KC (1991) Enzyme Microb Technol 13:296
18. Leckie F, Scragg AH, Cliffe KC (1991) Enzyme Microb Technol 13:801
19. Kim DI, Cho GH, Pedersen H, Chin CK (1991) Appl Microbiol Biotechnol 34:726
20. Su WW, Humphrey AE (1991) Biotechnol Lett 13:889
21. Jolicoeur M, Chavarie C, Carreau PJ, Archambault J (1992) Biotechnol Bioeng 39:511
22. Yokoi H, Koga J, Yamamoto K, Seike Y, Tanaka H (1993) J Ferment Bioeng 75:48
23. Zhong JJ, Yoshida M, Fujiyama K, Seki T, Yoshida T (1993) J Ferment Bioeng 75:299
24. Wang SJ, Zhong JJ (1996) Biotechnol Bioeng 51:511
25. Wang SJ, Zhong JJ (1996) Biotechnol Bioeng 51:520
26. Roberts SG, Shuler ML (1997) Curr Opin Biotech 8:154
27. Zhong JJ, Wang SJ, Wang HQ (1997) In: The Special Study Group for Biochemical Engineering (ed) Horizon of biochemical engineering. The Society of Chemical Engineers of Japan, Tokyo, p 175
28. Zhong JJ, Chen F, Hu WW (2000) Process Biochem 35:491
29. Zhong JJ, Bai Y, Wang SJ (1996) J Biotechnol 45:227
30. Bramble JL, Graves DJ, Brodelius P (1991) Biotechnol Bioeng 37:859
31. Liu S, Zhong JJ (1998) Process Biochem 33:69
32. Zhong JJ, Zhu QX (1995) Appl Biochem Biotechnol 55:241
33. Schlatmann JE, Koolhaas CMA, Vinke JL, ten Hoopen HJG, Heijnen JJ (1995) Biotechnol Bioeng 47:525
34. Martinez BC, Park CH (1993) Biotechnol Prog 9:97
35. Su WW, Humphrey AE (1990) Biotechnol Lett 12:793
36. Panda AK, Mishra S, Bisaria VS (1992) Biotechnol Bioeng 39:1043
37. Do CB, Cormier F (1991) Plant Cell Rep 9:500
38. Srinivasan V, Ryu DDY (1993) Biotechnol Bioeng 42:793
39. Zhang YH, Zhong JJ, Yu JT (1996) J Biotechnol 51:49
40. Liu S, Zhong JJ (1997) Enzyme Microb Technol 21:518
41. Liu S, Zhong JJ (1996) J Biotechnol 52:121
42. Zhong JJ, Wang DJ (1996) J Biotechnol 46:69
43. Fulcheri C, Morard P, Henry M (1998) J Agric Food Chem 46:2055
44. Murashige T, Skoog F (1962) Physiol Plant 15:473
45. Zhang YH, Zhong JJ (1997) Enzyme Microb Technol 21:59
46. Wang HQ, Yu JT, Zhong JJ (1999) Process Biochem 35:479
47. Fett-Neto AG, Melanson SJ, Nicholson SA, Pennington JJ, DiCosmo F (1994) Biotechnol Bioeng 44:967
48. Zhong JJ, Seki T, Kinoshita S, Yoshida T (1991) Biotechnol Bioeng 38:653

49. Kurata H, Seki M, Furusaki S, Furuya T (1991) J Chem Eng Jpn 24:783
50. Doran PM (1999) Biotechnol Prog 15:319
51. Singh G, Curtis WR (1994) In: Shargool PD, Ngo TT (eds) Biotechnological applications of plant cultures. CRC Press, Boca Raton, FL, p 151
52. Dunlop EH, Namdev PK, Rosenberg MZ (1994) Chem Eng Sci 49:2263
53. Ho CH, Henderson KA, Rorrer GL (1995) Biotechnol Prog 11:140
54. Hooker BS, Lee JM, An G (1989) Enzyme Microb Technol 11:484
55. Kieran PM, O'Donnell HJ, Malone DM, MacLoughlin PF (1995) Biotechnol Bioeng 45:415
56. MacLoughlin PF, Malone DM, Murtagh JT, Kieran PM (1998) Biotechnol Bioeng 58:595
57. Meijer JJ, ten Hoopen HJG, van Gameren YM, Luyben KChAM, Libbenga KR (1994) Enzyme Microb Technol 16:467
58. Takeda T, Kitagawa T, Takeuchi Y, Seki M, Furusaki S (1998) Can J Chem Eng 76:267
59. Kieran PM, MacLoughlin PF, Malone DM (1997) J Biotechnol 59:39
60. Steward N, Martin R, Engasser JM, Goergen JL (1999) Biotechnol Bioeng 66:114
61. Zhong JJ, Fujiyama K, Seki T, Yoshida T (1994) Biotechnol Bioeng 44:649
62. Leckie F, Scragg AH, Cliffe KC (1991) Biotechnol Bioeng 37:364
63. Kobayashi Y, Fukui H, Tabata M (1991) Plant Cell Rep 9:496
64. Gao J, Lee JM (1992) Biotechnol Prog 8:285
65. Schlatmann JE, Moreno PRH, Vinke JL, ten Hoopen HJG, Verpoorte R, Heijnen JJ (1994) Biotechnol Bioeng 44:461
66. Schlatmann JE, Vinke JL, ten Hoopen HJG, Heijnen JJ (1995) Biotechnol Bioeng 45:435
67. Zhong JJ, Seki T, Kinoshita S, Yoshida T (1992) Biotechnol Bioeng 40:1256
68. Su WW (1995) Appl Biochem Biotech 50:189
69. Rodriguez-Monroy M, Galindo E (1999) Enzyme Microb Technol 24:687
70. Pan ZW, Zhong JJ, Wu JY, Takagi M, Yoshida T (1999) Biotechnol Bioproc Eng 4:269
71. Ballica R, Ryu DDY, Powell RL, Owen D (1992) Biotechnol Prog 8:413
72. Curtis WR, Emery AH (1993) Biotechnol Bioeng 42:520
73. Zhong JJ, Yoshida T (1994) In: Ryu DDY, Furusaki S (eds) Advances in plant biotechnology. Elsevier Science, Amsterdam, p 255
74. Zhang YH, Wang HQ, Liu S, Yu JT, Zhong JJ (1997) Biotechnol Lett 19:943
75. Zhang YH (1996) MS thesis, East China University of Science and Technology
76. Zhong JJ, Yoshida T (1995) Enzyme Microb Technol 17:1073
77. Matsushita T, Koga N, Ogawa K, Fujino K, Funatsu K (1994) In: Ryu DDY, Furusaki S (eds) Advances in plant biotechnology. Elsevier Science, Amsterdam, p 339
78. Scragg AH, Ashton S, York A, Bond P, Stepan-Sarkissian G, Grey D (1990) Enzyme Microb Technol 12:292
79. Seki M, Takeda M, Furusaki S (1995) J Chem Eng Jpn 28:488
80. Seki M, Ohzora C, Takeda M, Furusaki S (1997) Biotechnol Bioeng 53:214
81. Phisalaphong M, Linden JC (1999) Biotechnol Prog 15:1072
82. Van Gulik WM, ten Hoopen HJG, Heijnen JJ (1992) Biotechnol Bioeng 40:863
83. Westgate PJ, Curtis WR, Emery AH, Hasegawa PM, Heinstein PF (1991) Biotechnol Bioeng 38:241
84. Kessler M, ten Hoopen HJG, Heijnen JJ, Furusaki S (1997) Biotechnol Tech 11:507
85. Asali EC, Mutharasan R, Humphrey AE (1992) J Biotechnol 23:83
86. Zhong JJ, Konstantinov KB, Yoshida T (1994) J Ferment Bioeng 77:445
87. Scheper TH, Lammers F (1994) Curr Opin Biotechnol 5:187
88. Taya M, Tone S, Prenosil JE (1989) Plant Tiss Cult Lett (Jap) 6:179
89. Ryu DDY, Lee SO, Romani RJ (1990) Biotechnol Bioeng 35:305
90. Tanaka H, Uemura M, Kaneko Y, Aoyagi H (1993) J Ferment Bioeng 76:501
91. Markx GH, Davey CL, Kell DB, Morris P (1991) J Biotechnol 20:279
92. Matanguihan RM, Konstantinov KB, Yoshida T (1994) Bioprocess Eng 11:213
93. Tanaka H, Aoyagi H, Jitsufuchi T (1992) J Ferment Bioeng 73:130
94. Zhong JJ, Fujiyama K, Seki T, Yoshida T (1993) Biotechnol Bioeng 42:542
95. Albiol J, Campmajo C, Casas C, Poch M (1995) Biotechnol Prog 11:88

96. Smith MAL, Reid JF, Hansen AC, Li Z, Madhavi DL (1995) J Biotechnol 40:1
97. Miyanaga K, Seki M, Furusaki S (2000) Biotechnol Bioeng 67:493
98. Yanpaisan W, King NJC, Doran PM (1998) Biotechnol Bioeng 58:515
99. Wen ZY, Zhong JJ (1995) Biotechnol Tech 9:521
100. Bailey CM, Nicholson H (1989) Biotechnol Bioeng 34:1331
101. Curtis WR, Hasegawa PM, Emery AH (1991) Biotechnol Bioeng 38:371
102. Hooker BS, Lee JM (1992) Biotechnol Bioeng 39:765
103. Van Gulik WM, ten Hoopen HJG, Heijnen JJ (1993) Biotechnol Bioeng 41:771
104. Glicklis R, Mills D, Sitton D, Stortelder W, Merchuk JC (1998) Biotechnol Bioeng 57:732
105. Schlatmann JE, ten Hoopen HJG, Heijnen JJ (1999) Biotechnol Bioeng 66:147
106. Bailey CM, Nicholson H (1990) Biotechnol Bioeng 35:252
107. Kurata H, Furusaki S (1993) Biotechnol Prog 9:86
108. Smith JM, Davison SW, Payne GF (1990) Biotechnol Bioeng 35:1088
109. Shioya S, Shimizu K, Yoshida T (1999) J Biosci Bioeng 87:261
110. Schlatmann JE, Nuutila AM, Van Gulik WM, ten Hoopen HJG, Verpoorte R, Heijnen JJ (1993) Biotechnol Bioeng 41:253
111. Scragg AH, Morris P, Allan EJ, Bond P, Fowler MW (1987) Enzyme Microb Technol 9:619
112. Biddington NL (1992) Plant Growth Regul 11:173
113. Kim DI, Pedersen H, Chin CK (1991) Biotechnol Bioeng 38:331
114. Schlatmann JE, Moreno PRH, Vinke JL, ten Hoopen HJG, Verpoorte R, Heijnen JJ (1997) Enzyme Microb Technol 20:107
115. Mirjalili N, Linden JC (1995) Biotechnol Bioeng 48:123
116. Wang HQ (1998) PhD thesis, East China University of Science and Technology
117. Pan ZW, Wang HQ, Zhong JJ (2000) Enzyme Microb Technol 27:714
118. Cho GH, Kim DI, Pedersen H, Chin CK (1988) Biotechnol Prog 4:184
119. Lee CWT, Shuler ML (1991) Biotechnol Tech 5:173
120. Songstad DD, Giles KL, Park J, Novakowski D, Epp D, Friesen L, Roewer I (1989) Plant Cell Rep 8:463
121. Stephanopoulos G (1994) Curr Opin Biotechnol 5:196
122. Shimizu K (2000) In: Zhong JJ (ed) Advances in applied biotechnology. ECUST Press, Shanghai, p 3
123. Verpoorte R, Van Der Heijden R, ten Hoopen HJG, Memelink J (1999) Biotechnol Lett 21:467
124. Shanks JV, Bhadra R, Morgan J, Rijhwani S, Vani S (1998) Biotechnol Bioeng 58:333
125. Srinivasan V, Ciddi V, Bringi V, Shuler ML (1996) Biotechnol Prog 12:457
126. Verpoorte R, Van der Heijden R, Memelink J (1996) Phytother Res 10:S12
127. Shinmyo A, Shoji T, Bando E, Nagaya S, Nakai Y, Kato K, Sekine M, Yoshida K (1998) Biotechnol Bioeng 58:329
128. Fussenegger M, Schlatter S, Daetwyler D, Mazur X, Bailey JE (1998) Nat Biotechnol 16:468
129. McCaskill D, Croteau R (1998) TIBTECH 16:349
130. Ebizuka Y, Kushiro T, Shibuya M (1999) Intl Ginseng Conf '99, Hong Kong
131. Zhong JJ (1999) Proceedings of the 5th Asia-Pacific Biochem Eng Conf. Phuket, Thailand
132. Kim SI, Choi HK, Kim JH, Lee HS, Hong SS (2001) Enzyme Microb Tech 28:202
133. Wang CG, Wu JY, Mei XG (2001) Biotechnol Prog 17:89
134. Stoner MR, Humphrey CA, Coutts DJ, Shih NJR, McDonald KA, Jackman AP (1997) Biotechnol Prog 13:799
135. Fowler MW (1992) In: Coombes JD (ed) New Drugs Mat Sources. IBC Tech Serv, London, p 131
136. James E, Lee JM (2001) this volume
137. Kutney JP (1996) Pure Appl Chem 68:2073
138. Wu J, Zhong JJ (1999) J Biotechnol 68:89

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# Gas Concentration Effects on Secondary Metabolite Production by Plant Cell Cultures

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One aspect of secondary metabolite production that has been studied relatively infrequently is the effect of gaseous compounds on plant cell behavior. The most influential gases are believed to be oxygen, carbon dioxide and other volatile hormones such as ethylene and methyl jasmonate. Organic compounds of interest include the promising antimalarial artemisinin (known as "qing hao su" in China where it has been a folk remedy for centuries) that is produced by *Artemisia annua* (sweet wormwood) and taxanes used for anticancer therapy that are produced by species of *Taxus* (yew). The suspension cultures of both species were grown under a variety of dissolved gas conditions in stoppered culture flasks and under conditions of continuous headspace flushing with known gas mixtures. An analysis is presented to show the culture conditions are such that equilibrium between the culture liquid and gas headspace is assured. The growth rate of the cells and their production rates of artemisinin and paclitaxel were determined. These and other parameters are correlated as functions of the gas concentrations. Interdependence of ethylene and methyl jasmonate is also explored with respect to regulation of secondary metabolite formation.

**Keywords.** Plant cell culture, Secondary metabolites, Oxygen, Carbon dioxide, Ethylene, Methyl jasmonate, Artemisinin, Paclitaxel, Engineering parameters



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# 1 Introduction

Plants provide a wide variety of biochemicals useful to humanity. Their uses include medicinal compounds, flavors, fragrances and agricultural chemicals. A number of investigators have studied the use of plant cells in culture, rather than whole plants, as sources of some of the more valuable organic compounds. Before such processes can become a viable manufacturing option, a great deal more must be learned about the optimum conditions for growth and productivity of cells in culture. The use of large quantities of individual cells in a controlled environment is the basis of the well-established fermentation industry. In most fermentation processes, microbial cells (bacteria or fungi) of a particular species are grown in large-scale suspension cultures. Conditions such as temperature, pH and concentrations of dissolved oxygen, carbon source and other nutrients are controlled in order to maximize the production rate of the desired compound. Productivities of commercial fermentation products have been enhanced orders of magnitude above those exhibited in nature. The same principles need to be applied to plant cell cultures.

## 1.1 Examples of Secondary Metabolites Produced by Plant Cell Culture

The production of commercial products from plant cell culture processes has been very slow to occur. Although the matter has been discussed in the scientific literature for well over three decades [1], only a few commercial-scale processes have even been attempted that use plant cell culture to produce secondary metabolites [2]. In Japan three submerged fermentation processes using plant cell cultures were developed by Mitsui Petrochemical Industries to produce berberine, ginseng and shikonin on scales from 4000 to 20,000 l. Only one of these, production of shikonin by suspension cultures *Lithospernum erythrorhizon*, can be considered a clear success. It has been operated since the early 1980s [3]. Difficulty in obtaining regulatory approval for use of these products as medicinals has impeded commercialization. In North America commercial production of sanguinarine and vanilla flavor was attempted but failed for re-

gulatory reasons. However, Phyton (Ithaca, NY) utilizes a 75,000-l reactor at a facility near Hamburg, Germany, to develop commercial production of paclitaxel [4].

In addition to food and fiber, plants are exploited for a large variety of commercial chemicals, including agricultural chemicals, pharmaceuticals, food colors, flavors and fragrances [5]. A few examples of plant-derived pharmaceuticals are digitalis (produced from *Digitalis purpurea*, prescribed for heart disorders), codeine (*Papaver somniferum*, sedative), vinblastine and vincristine (*Catharanthus roseus*, leukemia treatment) and quinine (*Cinchona officinalis*, malaria) [1]. It is believed that plant tissue culture production methods (called "phytoproduction") can be developed to profitably manufacture some of these chemicals [1–9]. Encouraged by these advantages, Routian and Nickell obtained the first patent for the production of substances by plant tissue culture in 1956 [10]. More recent patents cover many aspects of using plant cell culture for secondary metabolite production [11–13]. Numerous investigators have reported production of useful compounds in hairy root, callus and suspension cultures. The diversity of plant materials adaptable to culturing in hairy root and callus cultures has recently been reviewed [14, 15]. Suspension cultures of *Thalictrum minus* produced the stomachic and antibacterial berberine [16]. Callus cultures of *Stizolobium hassjo* produced the antiparkinsonian drug L-dopa [17]. Suspension cultures of *Hyoscyamus niger* L. produced a derivative of the anticholinergic hyoscyamine [18].

Some secondary metabolites have been observed in much higher concentrations in cultured cells than in whole plants of the same species. These include ginsengosides from *Panax ginseng* (27% of cell dry weight in culture, 4.5% in whole plants), anthraquinones from *Morinda citrafolia* (18% in culture, 2.2% in plants) and shikonin from *Lithospernum erythrorhizon* (12% in culture, 1.5% in plants) [19, 20].

Additional examples of substances synthesized by cell culture are listed in review articles and books [1–8, 14, 15, 21]. It is important to note that many of these compounds are secondary metabolites; that is, their production is not related to cell growth and division. Indeed, high rates of production of secondary metabolites usually occur during low rates of growth, often under conditions of significant physiological or biochemical stress on the cells [4].

## 1.2 Comparison with Well-Established Microbial Fermentations

Phytoproduction holds several difficulties compared with the well-established microbial fermentations [1, 4, 6, 22, 23]:

1 Plant cells grow much more slowly, with doubling times of the order of 40 h (compared with 0.3 h for some bacteria). Consequently, costs associated with cell generation are much greater.
2 Specific production rate tends to be lower. For example, despite several years of optimization studies, volumetric productivity of shikonin by suspension cultures of *L. erythrorhizon* was reported as 0.1 $g_{product}$ $l^{-1}$ $d^{-1}$ [23]. For com-

parison, the fungus *Penicillium crysogenum* yields 3.2 g $l^{-1}$ $d^{-1}$ of penicillin [24].
3 Plant cells may store their products in vacuoles rather than secrete them into the medium.
4 Plant cells are much more sensitive to shear forces than are bacteria or yeast cells, requiring much gentler aeration and agitation.

The medium in contact with cultured plant cells must consist of a large number of components for growth to occur. For the purpose of the present discussion, these components may be categorized as follows:

1 Water
2 Carbon source
3 Concentrated inorganic salts, including nitrogen sources
4 Trace salts (usually considered to be those of less than 0.1 g $l^{-1}$ in the medium)
5 Vitamins
6 Plant hormones and cytokinins
7 Medium conditioning factors (compounds produced in very small quantities by the cells themselves)
8 Dissolved gases

Clearly, the first six component types can be controlled during the initial formulation of the medium. It is these that have been the subject of optimization studies, which form a large part of the recent plant cell culture literature. The seventh category falls outside the capabilities of most investigators in the field. The concentrations of dissolved gases have also been neglected as components, possibly because they cannot be controlled in the same manner as dissolved solids.

### 1.3
### Importance of Dissolved Gases as Medium Components

In the present study, we attempted to determine the effects of important dissolved gases, oxygen, carbon dioxide and ethylene, on growth of species of three plant genera, *Nicotiana, Artemisia* and *Taxus*, in culture. The productivity of respectively valuable metabolic compounds, generic phenolic compounds, artemisinin and paclitaxel, and aspects of culture physiology were also studied. Artemisinin is a promising antimalarial drug and paclitaxel is an effective anticancer drug, so progress towards improved methods of manufacturing would be very desirable. Moreover, *N. tabacum, A. annua, T. cuspidata* and *T. canadensis* can be considered as model systems; the methods (and some of the conclusions) developed from the current studies may be applicable to other phytoproduction systems [25–30].

In an efficient commercial phytoproduction process, the cells will be in contact with near optimum concentrations of each dissolved gas. However, to insure that this occurs, the designer must know both the ideal concentrations and (for material balance reasons) the production and usage rates.

### 1.3.1
### *Specific Consumption of Oxygen*

Both the studies of Hulst et al. [31] and Hallsby [32, 33] reported zero-order consumption; that is, in the normal concentration range, cellular $O_2$ consumption was not dependent on its concentration. LaRue and Gamborg measured $O_2$ usage of 0.03 mmol $g_{dw}^{-1}$ $h^{-1}$ over the life of a culture of rose cells, but did not investigate the order of reaction [34].

Taticek et al. tabulated several values of maximum specific $O_2$ usage rates, which range from 0.2–0.6 mmol $g_{dw}^{-1}$ $h^{-1}$ [6]. Our experiments with *A. annua* suspension cell cultures indicate a maximum specific usage rate of 0.2 mmol $g_{dw}^{-1}$ $h^{-1}$. The wide variation in usage rates is suspicious; it may be an artifact of the variety of methods of estimation.

For comparison, several specific usage rates for industrially important bacteria and fungi are given. These range from 3.0 (*Aspergillis niger*) to 10.8 mmol $g_{dw}^{-1}$ $h^{-1}$ (*Escherichia coli*) [24].

### 1.3.2
### *Specific Productivity of Carbon Dioxide*

Thomas and Murashige investigated several solid callus cultures of several plant species, but did not test any suspension cultures [35]. Concentrations of $CO_2$, ethylene and some other gases were measured 24 h after flushing with air. Zobel measured $CO_2$ and $C_2H_4$ evolution from soybean callus cultures [36]. Fujiwara et al. measured $CO_2$ concentrations in closed vessels containing cultured plantlets [37]. None of these reports give enough information to permit the calculation of specific productivity.

### 1.3.3
### *Specific Productivity of Ethylene*

Ethylene ($C_2H_4$) is produced in essentially every part of every seed plant and affects a number of metabolic functions in very small concentrations. It is therefore considered a plant hormone [38]. Cultured plant cells are also known to produce $C_2H_4$.

The highest known ethylene release rate is by fading flowers of Vanda orchids, producing approximately $3 \times 10^{-3}$ mmol $g_{dw}^{-1}$ $h^{-1}$ [39]. In some of the sources mentioned in the previous section, both $CO_2$ and $C_2H_4$ concentrations were measured, but, again, not enough data is reported to obtain productivity values. LaRue and Gamborg report the amounts of $C_2H_4$ produced by suspension cultures of several species [34]. The time course data they present shows that $C_2H_4$ production is very unsteady; however, average productivities can be estimated. For soybean and rose cultures, for example, productivities were $1 \times 10^{-6}$ and $1.4 \times 10^{-5}$ mmol $g_{dw}^{-1}$ $h^{-1}$, respectively. Most of the other species tested lay between those results. Lieberman et al. measured ethylene productivities of callus and suspension cultures of apple [40]. For callus cultures, productivity peaked at $2.1 \times 10^{-6}$ mmol $g_{dw}^{-1}$ $h^{-1}$, and averaged about two-thirds that value. For

suspension cultures, productivity peaked at $9.4 \times 10^{-6}$ mmol $g_{dw}^{-1}$ $h^{-1}$, but averaged $2.7 \times 10^{-6}$ mmol $g_{dw}^{-1}$ $h^{-1}$.

## 1.4 Review of Relevant Engineering Parameters

In a review article advocating entrapped plant cell cultures, Shuler et al. [41] point out the problem in large-scale suspension cultures of maintaining sufficient oxygen transfer without excessive mechanical shear on the cells. A process designer must not overdesign for oxygen transfer because this would result in increased cell damage. Conventional bioreactors are completely back-mixed stirred tank reactors (STR) that deliver oxygen and remove carbon dioxide by sparging air through the medium. Conventional STR bioreactors exhibit oxygen transfer rates (OTRs) of between 5–150 mmol $O_2$ $h^{-1}$ $l^{-1}$ under normal operating conditions. When the growth-limiting nutrient is oxygen in batch culture, cells grow exponentially until the oxygen uptake (or consumption) rate (OUR) exceeds the oxygen delivery rate (OTR) of the bioreactor. The point at which oxygen becomes the limiting nutrient and the OTR dictates the growth rate can be illustrated at various OTRs using the exponential growth and yield expressions for growth:

$$x_t = x_0 \, e^{\mu t} \tag{1}$$

$$\Delta x = Y_{x/s} \, \Delta S \tag{2}$$

where $\mu$ is the specific growth rate ($h^{-1}$) (which is constant until such time that nutrient becomes limiting, a product becomes toxic, or culture conditions change), $t$ is time (h), $x$ is cell dry weight (g $l^{-1}$) at the initial condition, 0, and after time $t$, $Y_{x/s}$ is the yield coefficient, and S is substrate concentration (g $l^{-1}$).

### 1.4.1 *Mass Transfer Considerations in Entrapped Culture Systems*

Oxygen limitation is of concern in entrapped as well as suspension cultures; for example, many investigators have found it necessary to guarantee sufficient aeration by installation of oxygen-permeable silicone tubing (with oxygen pressure on the inside of the tube) in immobilized cell bioreactors. The $O_2$ flux rate is calculated and judged more than sufficient for the entrapped cells, but no estimate of the actual requirement was presented. In multi-phase bioreactors resistance to oxygen transfer is the sum of resistances in the gas-phase viscous boundary layer, the liquid-phase viscous boundary layer and the immobilized cell surfaces. Generally, however, in cases of several resistances in series, only one of the resistances is large enough (relative to the others) to control the transport rate and is referred to as the rate-limiting resistance. A Wilson plot (reciprocal of mass transfer coefficient vs. the reciprocal of linear fluid velocity) can be used to determine which is the rate-limiting resistance to oxygen transfer. Extrapolation of such double reciprocal plots to the y-axis yield mass transfer rates at infinite velocity, where there would be no boundary layer resistance.

Significant effort on immobilized plant cell [9, 25, 42] and transformed hairy root [43–45] cultures has been expended by several groups of chemical engineering researchers. The growth of *Agrobacterium*-transformed "hairy root" cultures of *Hyoscyamus muticus*, examined in various liquid- and gas-dispersed bioreactor configurations, has been studied by McKelvey et al. [46]. Accumulated tissue mass in submerged air-sparged reactors was 31 % of that in gyratory shake-flask controls. Experiments demonstrated that poor performance of sparged reactors was not due to bubble shear damage, carbon dioxide stripping, settling or flotation of roots. Impaired oxygen transfer due to channeling and stagnation of the liquid phase was the apparent cause of poor growth.

Hulst and co-workers obtained a value for the $O_2$ uptake in both suspended and calcium alginate immobilized *Daucus carota* (carrot) cells [31]. This value was approximately $3 \times 10^{-7}$ mol $g_{dw}^{-1}$ $s^{-1}$. Nevertheless, the value of Hallsby's estimate of uptake of oxygen, $3 \times 10^{-10}$ $g_{dw}^{-1}$ $mol^{-1}$ $s^{-1}$ for *N. tabacum*, will be used [32, 33]. Hulst's observation that specific consumption was similar for suspended and immobilized cells is assumed to apply [31].

During most of our studies with calcium alginate immobilized *N. tabacum* cells, the bioreactor columns contained about 0.04 g (dry weight) of cells; of course, this value varied from test to test. It is reasonable to assume that the feed medium was saturated in $O_2$. The $O_2$ mole fraction, $x$, in the medium (at saturation) is given by Henry's Law [47]:

$$x = p/H \tag{3}$$

where p is the partial pressure of $O_2$ in the aeration air (in atmospheres) and H is Henry's Law constant, reported at 25 °C to be $4.4 \times 10^4$ (in units consistent with $x$ and p). The latter value is nearly independent of pressure. The atmospheric pressure of our laboratory (and hence the $O_2$ partial pressure in the aeration air) is affected by its elevation, approximately 1500 m above sea level. The average total pressure is 846 millibars, or 0.835 atmosphere [48]. Air is 21 % $O_2$, so the $O_2$ partial pressure in the saturated medium,

$$x = 0.835 \text{ atm} \times 0.21/(4.4 \times 10^4 \text{ atm mole fraction}^{-1}) = 4.0 \times 10^{-6} \text{ [mole fraction } O_2\text{]}.$$

Converting from mole fraction to concentration units by multiplying by the molar concentration of liquid water, the $O_2$ concentration is:

$$4 \times 10^{-6} \times 0.056 \text{ mol cm}^{-3} = 2.2 \times 10^{-7} \text{ mol cm}^{-3}$$

The flow rate for most tests was of the order of 0.5 $cm^3$ $h^{-1}$. This resulted in a total feed rate of $O_2$ of $3.1 \times 10^{-11}$ mol $s^{-1}$, less by a factor of 10 than the estimated requirement.

Suppose it is correct that $O_2$ consumption is a zero-order process until its concentration is depleted to 15 % of saturation (at sea level). Then 80–85 % of the available oxygen was consumed by approximately 10 % of the cells – the 10 % with which the feed first came in contact. Clearly, the remainder of the cells was starved for oxygen. The resulting biochemical stress could have effected an increase in productivity of phenolics; indeed, Hallsby concluded from batch ex-

periments that a suboptimum level of $O_2$ may be advantageous for the formation of extracellular phenolics [31]. However, it is likely that most cells were severely restricted in metabolism (both primary and secondary) or died due to lack of oxygen. The decline in productivity that we observed suggests death of most of the cells, although it is surprising (and not conclusively explained) that this decline occurred over such a long period. One would have expected that the productivity would drop rapidly to 10% of the initial value and remain there.

The following discussion centers on the particle effectiveness factor of calcium alginate immobilized cells. Other investigators have estimated the effective diffusivity of oxygen in this material. This parameter can have, as will be shown, a significant effect upon whether any of the cells are starved for oxygen. If sufficient $O_2$ had been supplied to the alginate beads, there would not have been diffusion limitation in the beads, as evidenced by the following. First, it will be shown that the oxygen concentration in the medium at the bead surface very nearly equals the bulk concentration. Then it will be shown that this concentration is sufficient to achieve an effectiveness factor of unity for the zero-order process believed to occur.

The steady-state diffusion rate from a stagnant medium of infinite extent to a sphere is given by:

$$\text{Flux} = (2D/d)\,(c_B - c_o) \tag{4}$$

where $D$ is the diffusivity in the bulk fluid, d is the bead diameter, and $c_o$ and $c_B$ are the concentrations of the diffusing species at the bead surface and in the bulk liquid, respectively [49]. Diffusive flux is the rate of flow of the species through the surface per unit area. Thus, at steady state, the product of the flux and the surface area equals the consumption rate within the bead. This equality is:

$$(\pi d^2)\,(2D/d)\,(c_B - c_0) = (d^3\pi/6)\;{}_b k \tag{5}$$

where the right side represents the bead volume times the biomass density times the consumption rate per unit biomass. Rearranging,

$$c_B - c_o = (d^2\;{}_b k)/12D \tag{6}$$

The approximate parameter values are: $d = 0.5$ cm, ${}_b = 2 \times 10^{-4}$ $g_{dw}$ $cm^{-3}$, k = $8 \times 10^{-9}$ mol $g_{dw}^{-1}$ $s^{-1}$, and $D = 2.4 \times 10^{-5}$ $cm^2$ $s^{-1}$, giving:

$$c_B - c_o = 1.4 \times 10^{-9} \text{ mol cm}^{-3}.$$

This compares to a $c_B$ of $2 \times 10^{-7}$ mol $cm^{-3}$, so the surface concentration is within 1% of the bulk liquid concentration.

For a zero-order process, the effectiveness factor (defined as the ratio of the actual consumption of reactant to that which would occur in the absence of mass transfer resistance) is equal to unity whenever the following inequality holds:

$$d < (24\,D_{eff}c_o/k_b)^{1/2} \tag{7}$$

Here $d$ is the bead diameter (cm), $k$ is the intrinsic rate constant (moles consumed/s per gram dry weight of biomass), $b$ is the biomass per bead volume

($g_{dw}$ $cm^{-3}$), $D_{eff}$ is the effective diffusivity ($cm^2$ $s^{-1}$) of oxygen in a bead, and $c_o$ is the $O_2$ concentration (mol $cm^{-3}$) at the bead exterior. Hulst estimates the effective diffusivity of $O_2$ in a calcium alginate bead to be $1.9 \times 10^{-5}$ $cm^2$ $s^{-1}$ [31]. Near the inlet of the column, $c_0 = 2 \times 10^{-7}$ mol $cm^{-3}$ (see above). The rate constant $k$ is estimated as $8 \times 10^{-9}$ mol $g_{dw}^{-1}$ $s^{-1}$ (this is Hallsby's consumption estimate as used above). The biomass per bead volume was typically $2 \times 10^{-4}$ $g_{dw}$ $cm^{-3}$. The critical diameter for these conditions is 7.5 cm. Even if there are large uncertainties in the rate parameters, the critical diameter is much larger than the actual diameter of 0.5 cm.

### 1.4.2 *Dissolved Gases in Suspension Culture Systems*

As mentioned in the previous section, at least two investigators observed zero-order dependence of $O_2$ consumption on its concentration. Hallsby claims this is true for suspended tobacco cells whenever $O_2$ concentration exceeds 15% of the concentration in water in equilibrium with air (the "air saturation concentration") [33]. Hulst et al. assert that experimental values for $K'_m$ (the $O_2$ concentration at which $O_2$ usage is half the usage at very high concentrations) are between 2 and 20% of air saturation [31]. At concentrations much above $K'_m$, consumption would be zero order.

If $O_2$ consumption were indeed zero order for a particular plant species, then it would appear that any phytoproduction process involving that species would require only that a minimum dissolved $O_2$ concentration be maintained; any concentration increase beyond that would be irrelevant. In the case of tobacco cells, any concentration greater than 15% of air saturation would yield the same metabolic rate and, presumably, the same productivity of all metabolites. If, on the other hand, consumption is first order in the concentration range achievable in a practical bioreactor (equivalently, if $K'_m$ is comparable to working concentrations), then its concentration is an important control parameter in the bioreactor. However, Kobayashi et al. studied berberine production by suspended and immobilized cells of *Thalictrum minus* [50]. They assert that $O_2$ uptake is a zero-order process but observed that berberine production depended on $O_2$ availability. They controlled that availability by adjusting the speed of shaking of the culture flasks, thus varying the mass transfer coefficient for absorption of $O_2$.

Tate and Payne fed controlled concentrations of $O_2$ and $CO_2$ through the headspaces of suspension cultures of *Catharanthus roseus*, and reported a half-saturation constant for growth (the $O_2$ concentration at which growth was half that occurring at abundant $O_2$) of 16% of air saturation [51]. They also observed suppressed growth at $O_2$ concentrations above 70% in the gas phase (350% of air saturation) but did not observe that phenomenon in *Daucus carota* cells. Su and Humphrey grew *Anchusa officinalis* in a "perfusion bioreactor", a fermentor in which an oxygen/air mix was fed through microporous tubing rather than by bubbling [52]. They plotted dissolved $O_2$ concentration (minimum of 6% air saturation) and reported that this concentration remained "at a non-limiting level".

Carbon dioxide is, of course, fundamentally important to plants because of photosynthesis. Most plant cell cultures are heterotrophic, non-photosynthetic and use a chemical energy source. It is reasonable to suspect, however, that some of the control mechanisms for the photosynthetic dark reactions would be regulated by $CO_2$ concentration. This could affect both cell growth and, indirectly, production of useful compounds. More concretely, $CO_2$ is known to promote synthesis of ethylene [38]; on the other hand, $CO_2$ concentrations of 5–10% inhibit many ethylene effects [53].

A few studies on cultured plant cells have shown significant effects of $CO_2$. Using an airlift fermenter, Fowler observed that supplying $CO_2$ to a tobacco cell culture increased biomass growth [54]. Maurel and Paeilleux found the same using *Catharanthus roseus* cells [55]. Stuhlfauth et al. found it increased secondary metabolite production in *Digitalis lanata* [56]. In headspace gas fed shake flasks, Tate and Payne also looked for an effect of $CO_2$ concentration on cell growth and found none [51]. Kim et al. grew *Thalictrum rugosum* cells in an airlift fermenter and found that addition of $CO_2$ to the air feed had no effect on cell growth but increased formation of berberine, the desired product [57].

A significant concern involves aerated systems, as one would expect to use in a large-scale process [58]. Preliminary studies typically involve growth in small shake flasks with limited gas exchange. When the process is scaled up to an aerated bioreactor, the aeration may strip volatile compounds, especially $CO_2$ and $C_2H_4$, produced by the cells and which are necessary to some extent for growth and productivity. If changes in performance occur upon scale-up, it may be difficult to determine the cause.

Ethylene has been shown to have a variety of effects on living plants. Thirty-five distinct functions were tabulated by Abeles, based on literature reports existing in 1973 [59]. The majority of cases showed threshold $C_2H_4$ activity at 0.01 ppm, half-maximal effect at 0.1 ppm, and saturation (that is, further increase in $C_2H_4$ concentration did not increase the effect) at about 10 ppm. The parts per million concentrations Abeles reports are the gas-phase concentrations. In most cases, there was no further effect beyond the saturation concentration, although there were a few instances of the effect being reversed at higher concentrations [28, 60].

A number of investigators have added ethylene to plant cell cultures to enhance the yield of desired compounds. Bagratishvili and Zaprometov periodically added small quantities of 2-chloroethylphosphonic acid (commonly called ethephon, trade name Ethrel) to suspension cultures of *Camellia sinersis* [61]. In aqueous solution, ethephon breaks down to release ethylene, HCl and phosphate. Growth was reduced by 20% but production of phenolics and flavans increased by 90 and 75%, respectively. Songstad et al., studying sanguinarine production by suspension cultures of *Papaver somniferum*, observed an increase in $C_2H_4$ production occurring at the same time as sanguinarine production [62]. This led them to add Ethrel to test cultures, but it did not enhance productivity. Kobayashi et al. produced berberine in suspension cultures of *Thalictrum minus* and enhanced production by addition of Ethrel but reduced it greatly by addition of silver thiosulfate, a known potent inhibitor of $C_2H_4$ activity [63]. Kim et al. grew the same organism in an airlift bioreactor and improved berbe-

rine productivity by addition of both $CO_2$ and $C_2H_4$ to the gas feed [57]. The same group grew cultures in numerous shake flasks, allowing them to try several combinations of several added substances including Ethrel [64]. For example, their most effective combinations involved Ethrel and $CuSO_4$ addition and a two-stage culture with high sucrose concentration in the production stage. Cho et al. enhanced production of purine alkaloids by *Coffea arabica* cells by addition of Ethrel and other strategies [65].

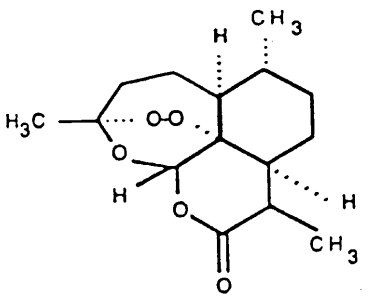
## 2 *Artemisia annua* Production of Artemisinin: Dependence on Dissolved Gas Concentrations

Malaria is one of the world's most serious human health problems. According to the World Health Organization, more than 200 million new infections occur each year, many resulting in death [66]. The disease is caused by protozoa of the genus *Plasmodium*, most notably *P. falciparum*, which live in the intestines of female *Anopheles* mosquitoes. Humans are infected by bites from infected mosquitoes, by blood transfusions from infected donors, or by an expectant mother transmitting the disease to her child. Malaria is endemic in Sub-Saharan Africa, Central and South America, the Indian subcontinent and Oceania [67].

For many years, the treatment of choice for malaria has been chloroquine. Unfortunately, chloroquine-resistant strains of *Plasmodium* species have developed, emphasizing the need for new antimalarials. One promising antimalarial is artemisinin.

Beginning in 1967, the government of the People's Republic of China conducted a systematic study of native plants used as folk remedies for a variety of ailments [67]. The herb known to the Chinese as "qing hao" (*Artemisia annua*, commonly called sweet wormwood or annual wormwood) was first mentioned in writings from 168 BC as a remedy for hemorrhoids. Since then, it has been recommended to relieve fevers (in 340 AD) and specifically for malaria symptoms (in 1596). In the 1970s, the activity against malaria was confirmed; the active ingredient was isolated and its structure identified. It was given the name "qing hao su" ("active ingredient in qing hao") and in the West is now known as artemisinin.

Artemisinin is a sequiterpene lactone with an endoperoxide bridge. Concentrations in plant biomass as high as 0.5% (weight of artemisinin divided




**Structure 1.** Artemisinin

by weight of dried leaves or flowers) were obtained from *A. annua* plants growing in the Sichuan province of China. *A. annua* grows wild in many places, including the United States, but artemisinin contents in plants outside of China tend to be much lower.

Thousands of patients in China and Myanmar (formerly Burma) have been successfully treated but, as of 1990, artemisinin is unavailable elsewhere. Relatively low toxicity has been observed, along with substantial effectiveness. Unfortunately, the relapse rate is high. Nevertheless, use of artemisinin or its derivatives in conjunction with other treatments is considered promising [67].

The small concentrations of artemisinin in plant material (and the possibility of an embargo of Chinese plant material) has led several groups of researchers to investigate use of plant cell cultures as a source of artemisinin [26, 68–70]. The results have generally been disappointing and tended to reflect some of the problems of other phytoproduction studies. Nevertheless, the results of such studies, including this one, can guide the development of phytoproduction using this and other plant species.

## 2.1 Initial Specific Gas Usage and Productivities

Eighteen cultures of *A. annua* were subjected to stoppered culture tests, which will be described below. The intention was to determine the specific productivities of $CO_2$ and consumption rate of $O_2$ under various conditions.

To obtain the "initial" rate of formation (or usage) of any gas, its change in gas-phase concentration over the first 24 h of the test (or as near as permitted by the analysis times) was divided by the estimated biomass (dry weight basis) during that period and by the time period. The actual equation along with its development will be presented below. Ideally, we would like to obtain the instantaneous rate (of usage or production) as soon as the rubber stopper is applied. As soon as the gas composition in the headspace (hence also, the concentration of dissolved gases in the medium) changes, the behavior of the cells starts to change. However, there is, of course, uncertainty in measurement of the gas concentrations. If samples taken at a short time interval were used in the productivity calculation, the subtraction of two uncertain values close to each other would make the calculated productivity unacceptably inaccurate. The 24-h period was chosen to obtain a period of relatively large change in gas concentrations while not *drastically* altering the metabolic conditions during the period of the rate calculation.

The quantities of gaseous $CO_2$, $O_2$ and $C_2H_4$ in the headspace of the stoppered culture flask were calculated from the measured concentrations. The quantity of each dissolved gas in the liquid can be estimated as described below using Henry's Law. The total volume of the stoppered flask is 550 ml. The volume $V_l$ of liquid and cells is 83 ml, leaving 467 ml of headspace ($V_g$). The atmospheric pressure (P) at Fort Collins, Colorado, where the experiments took place, is roughly 0.835 atm; any change in pressure inside the flask after the start of the experiment is negligible. Temperature ($T$) in our laboratory averaged

298 K. The total amount ($N$, in moles) of headspace gas in each culture can be determined from the Ideal Gas Law.

$$N = (PV_g)/RT \tag{8}$$

$= [(0.835 \text{ atm})(.467 \text{ l}) ]/[(0.082 \text{ l atm mol}^{-1} \text{ K}^{-1})(298 \text{ K})]$
$= 0.0160$ moles

The total number of moles, $N_i$, of species $i$ in the headspace is given by

$$N_i = 0.0160\, y_i \tag{9}$$

$y_i$ being the measured headspace mole fraction of $i$.

It is necessary to estimate the quantity of each gas in the liquid to accurately determine the productivities and usage rates. The species' equilibrium concentration, $x_i$, in the liquid is estimated by Henry's Law (Eq. 3). Unfortunately, $H$, the Henry's Law constant, for a gas in contact with a solution depends on the nature and concentrations of dissolved solids, tending to be less than the value for pure water [71]. For this reason, we can only obtain an upper limit for the dissolved gas quantity. However, the solubility depression for our rather dilute culture medium is low. A 0.5 mole/l concentration of sodium chloride results in an oxygen solubility depression of 15% [71]. The total concentration of dissolved solids in our medium was less than half of that (0.22 mole/l), so the gas solubility depression was almost certainly less than 10%. A more serious uncertainty occurs because the culture volume includes cell volume; by treating the entire 83 ml as liquid volume ($V_l$), we may tend to overestimate the dissolved gas quantity.

The quantity ($x_i V_l$) is the number of moles of $i$ dissolved in the liquid. By combining Eqs. (8) and (9), and using the known values of $P$ and $V_l$, we get:

$$(x_i V_l) = P y_i V_l/H = N_i(4.33 \text{ l atm mol}^{-1})/\text{H} \tag{10}$$

For $CO_2$, $H$ is approximately 31.6 mol $l^{-1}$ $atm^{-1}$ [47], so

$$(x_{CO2} V_l) = 0.14\, N_{CO2} \tag{11}$$

Aqueous $CO_2$, unlike the other gases we will consider, can further react to form other species, $H_2CO_3$, $HCO_3^-$ and $CO_3^{2-}$. The relative concentrations of the four aqueous species are a function of pH [72]. The pH of the *A. annua* cultures remained below 6.0. Under these conditions, the ratio of $[HCO_3^-]$ to the sum of $[H_2CO_3]$ and $[CO_2]$ remained less than 0.43; $[CO_3^{2-}]$ was negligible (the brackets indicate liquid phase concentration). The total value of the number of moles of dissolved $CO_2$ and related species was always, therefore, less than 20% of $N_i$ (14% plus 0.43 × 14%). For lack of a more precise estimate, we calculate the total $CO_2$ in both phases as 1.2 times that in the gas phase.

It turns out that the other gases, $O_2$ and $C_2H_4$, are less soluble so their liquid-phase quantities may be neglected in comparison to the gas phase. For $O_2$, $H = 790$ atm l $mol^{-1}$, so $x_{O2} V_l = 0.005 \times N_{O2}$. For $C_2H_4$, $H = 205$ atm l $mol^{-1}$, so $x_{C2H4} V_l = 0.021 \times N_{C2H4}$ [47]. Clearly, the liquid-phase quantities of both of these can by neglected compared to the gas phase.

The rate quantity of interest for each gas species is the specific productivity (for $O_2$, the specific usage rate), i.e. the amount of gas produced (or used) per

unit time per unit biomass. The amount of biomass at any given time is estimated from the initial and final biomass by assuming that growth is proportional to oxygen usage. Thus,

$$X(t) = X(0) + \frac{(\%O_2(0) - \%O_2(t))}{(\%O_2(0) - \%O_2(f))} \cdot (X(f) - X(0)) \tag{12}$$

Here, $X(t)$, $X(0)$ and $X(f)$ are the quantities of biomass (dry weight) at time $t$, the start of the test and the finish of the test, respectively. Similarly, $\%O_2(t)$, $\%O_2(0)$ and $\%O_2(f)$ are the measured $O_2$ percentages in the headspace at those times. This calculation could not be performed for the tests started with cultures that had grown under normal closures before being flushed and stoppered, since the biomass at the start of the test was unknown. The $X(t)$ values for those tests were estimated by working backwards from their final biomass, assuming that the ratio of (biomass generated)/($O_2$ consumed) was similar to those of the other tests. The $X(t)$ values for the 7- and 8-day-old cultures are consequently much less reliable.

The initial specific $O_2$ usage rate, $\sigma_{O2}$ (in millimoles per hour per gram of dry biomass), is thus estimated as:

$$\sigma_{02} = \frac{(\%O_2(0) - \%O_2(t))}{(t\text{-}0) \cdot X(t/2)} \cdot 0.160 \tag{13}$$

where $X(t/2)$ is the average biomass quantity over the initial period. The constant comes from the total quantity of headspace gas, 16 millimoles, corrected for the use of percentages (rather than mole fractions) in the numerator of Eq. (13). The calculations for $CO_2$ and $C_2H_4$ are fully analogous except:

a Those two gases are *produced* so the final concentration comes first in the subtraction.
b In the calculation for $CO_2$ one must further multiply the result by 1.2 to account for dissolved gas (as explained above).

The above development has tacitly assumed equilibrium for each species between the phases and also among the dissolved species containing $CO_2$. The reasoning presented below is intended to justify this. Each molecule of $CO_2$ measured in the gas phase must go through the following steps: synthesis and export by the cells, reaction to $H_2CO_3$, reaction to $CO_2$ and mass transfer from the liquid to the gas phase. The synthesis and transport (taken here as one process) is by far the slowest (hence, rate-determining) step, as is shown below. It is assumed that the $CO_2$ is produced by the cells in the form of $H_2CO_3$, $HCO_3^-$, or $CO_3^{2-}$; for our purposes, it does not matter which. The chemical reactions between $H_2CO_3$, $HCO_3^-$ and $CO_3^{2-}$ are known to be faster than that between $H_2CO_3$ and $CO_2$ [73].

Consider the rate expressions for the forward steps as described in the previous paragraph:

1 Biomass produces carbonate:

$$r_{H2CO3} = k_f\,[\text{cell mass}] \tag{14}$$

2 $H_2CO_3 \rightarrow CO_2 + H_2O$:

$$r_{CO2} = k_{CO2}\,[H_2CO_3] \tag{15}$$

3 $CO_2$(aqueous) $\rightarrow$ $CO_2$(gas):

$$r_{deabs} = k_l a\,[CO_2] \tag{16}$$

Since the object of the exercise is to determine how quickly equilibrium is established, we are only concerned with the time before the reverse reactions. Here the first two values of $r$ are the rates of formation (in, for instance, millimoles of species per liter per hour). The quantity $r_{deabs}$ is the rate of deabsorption of $CO_2$, in the same units as the other two rates. The brackets indicate liquid-phase concentration and the coefficients of the bracketed terms are the respective rate constants, which will be described below. If any of the rate constants is much lower than the others, that step is the slowest, therefore rate-determining, step. As will be demonstrated below, this is indeed the case.

The value of $k_{CO2}$ is 20 $s^{-1}$ (or 7200 $h^{-1}$) at room temperature [73]. The value of $k_l a$, the interphase mass transfer coefficient, for our system must be estimated based on literature reports of mass transfer rates in relatively similar systems. Although the estimate of $k_l a$ obtained below is highly uncertain, it is orders of magnitude less than $k_{CO2}$ but very much larger (hence, less rate-determining) than $k_f$.

## 2.2 Absence of Significant Mass Transfer Resistance

The mass transfer resistance at a liquid-vapor interface results from two resistances, the liquid boundary layer and the gas boundary layer. In conditions involving water and sparingly soluble gases, such as occurs here, the liquid-phase resistance is almost always predominant [71]. For this reason, equation (16) involves only $k_l$, the mass transfer coefficient across the liquid boundary, and a, which is the gas bubble surface area per unit volume of liquid. Often, as here, those factors cannot be estimated individually, so $k_l a$ is treated as a single parameter.

The applicability of the O'Connor–Dobbins correlation [73] can be verified using data from Kobayashi et al. [50] They report placing 30-ml volumes of medium in 100-ml Erlenmeyer flasks on a reciprocal shaker at 100 strokes/min. They do not report the stroke length, but a 1-inch stroke is a reasonable guess. Under such conditions, the average liquid depth was roughly 1.3 cm, velocity was 8.5 cm/s, and $D = 2.4 \times 10^{-5}$ $cm^2/s$ (they were measuring $K_l a$ for $O_2$). The O'Connor–Dobbins correlation gives $k_l a = 19.5$ $h^{-1}$.

Kobayashi et al. [50] measured $K_l a$ (capital $K$ because they measured the global mass transfer coefficient) to be 17.5 $h^{-1}$, certainly close enough to the correlation to justify the latter's use as a reasonable approximation.

The results of stoppered culture tests that we conducted using *A. annua* suspension cell cultures in shake flasks are used to evaluate $k_f$, the formation rate constant for dissolved carbonate from biomass. Our experimental values ranged from 0.1 to 0.25 millimoles $CO_2$ $g_{dw}^{-1}$ $h^{-1}$ of biomass. Since the biomass quan-

tity never exceeded 0.8 g in any of the tests, the production rate never exceeded 0.2 millimoles $h^{-1}$ (the arithmetic product of the maximum productivity and the maximum biomass). This rate was enough to change the headspace gas composition to 1.25% (the approximate uncertainty of measurement of gas-phase composition) per hour. Thus, in the time it took for the composition to change by an amount barely detectable, nearly 20 time constants of mass transfer occurred. During each time constant, the difference between equilibrium and actual concentration diminished by a factor of 1/e (e being the base of the natural logarithms, approximately 2.72). The lag due to mass transfer resistance was very much less than the uncertainty in the measurement.

Similar calculations show that the $O_2$ and $C_2H_4$ concentrations were sufficiently close to equilibrium to warrant neglecting any mass transfer resistance. In our culture experiments in which headspace gas concentrations were kept constant by the flow of defined gas mixtures of $O_2$, $CO_2$ and $C_2H_4$ through the headspace of the cultures (gas feed tests) [27–30], the same processes of $H_2CO_3$ production, reaction to $CO_2$ and deabsorption of $CO_2$ (governed by Eqs. 14–16) occur. The rate constants $k_{CO2}$ and $k_l a$ should be identical in the gas feed tests to those in the stoppered culture tests that are discussed below. The value of $k_f$ probably varies as a function of the dissolved gas composition. However, its value would have to be at least an order of magnitude greater for the carbonate formation rate *not* to be the rate-determining step. Since this is implausible, it is quite unlikely that dissolved $CO_2$ concentration in the culture medium of the gas feed tests would be any different than that in equilibrium with the headspace gas. Again, the same reasoning would apply to the $O_2$ and $C_2H_4$ concentrations.

### 2.3
### Calculation of Specific Oxygen Usage Rate Throughout a Test

The headspace gas concentrations of the stoppered cultures were measured periodically until they stopped changing significantly. In particular, the consumption of $O_2$ was followed as a marker of cellular activity. Oxygen consumption, rather than $CO_2$ production, was chosen as an indicator of metabolic activity, for three reasons. First, the total amount of $O_2$ in solution was much less than the amount in the headspace so any uncertainty in the former quantity was an insignificant fraction of the total amount of $O_2$. Second, production of $CO_2$ can occur due to both aerobic and anaerobic metabolism. The latter process, noticeable under reduced $O_2$ concentrations (the Pasteur effect), results in less growth but more sugar usage and much more $CO_2$ production than does the aerobic process [38]. Use of $CO_2$ output as an activity indicator could exaggerate the activity occurring at low oxygen concentration. Third, because of relative concentration differences, the analysis of $O_2$ concentration was more accurate than that of $CO_2$.

The gradual reduction in cellular activity, culminating in culture death a few days after cultures were stoppered, was clearly the result (directly or indirectly) of changes in dissolved gas concentrations. Otherwise, the cells would have continued actively metabolizing as long as did the conventional unstoppered cultures. The conventional cultures, capped with permeable foam and foil, con-

tinued growing at least 13 days [74]. The *simplest* explanation of declining activity is the declining $O_2$ concentration.

The following series of steps was performed for each stoppered culture test in order to obtain a plot of specific oxygen usage as a function of oxygen concentration. The $O_2$ concentration at each time was converted to total millimoles $N_{O2}$ of $O_2$ in the headspace. In each test, a suspension culture of *A. annua* was flushed with a gas mixture of known composition, then sealed and placed on a rotary shaker. The gas headspace was sampled at several hour intervals; the measured concentrations from all the tests were used to calculate the $O_2$ consumption and $CO_2$ production rates.

### 2.4 Effect of Headspace Composition on Growth and Production of Artemisinin

Four types of stoppered culture tests were performed as follows:

a New culture from 21-day-old inoculum, flushed with air, shaken at 95 rpm
b Same as 'a' except flushed with high $CO_2$, high $O_2$ gas mixture
c Same as 'a' except tests were started with 7- or 8-day-old cultures
d Same as 'a' except inoculum was from a 10-day-old culture

The initial specific rates of $O_2$ consumption and $CO_2$ production are calculated and interpreted, and the effect of diminishing $O_2$ concentration on its specific consumption rate is investigated. For each test type, the mean and standard deviation of the $O_2$ and $CO_2$ specific rates are calculated and tabulated in Table 1. The four types of stoppered culture tests were compared using two-sample t tests on these quantities to determine whether the particular conditions affected the cell metabolism [74]. If either initial rate showed a statistically significant (95% confidence level) difference between the two test types being compared, then we concluded that the conditions affected metabolism and would be likely to affect bioreactor performance.

Table 2 shows the comparisons made and the probability that the difference between the sets of data would occur if the sets were actually from the same population. The results and their interpretations follow:

1 a vs. b (effect of flushing with air vs. flushing with a high $CO_2$, high $O_2$ gas mixture):

**Table 1.** Summary of gas exchange data from stoppered culture tests using *A. annua*. Mean initial specific rates of $O_2$ usage and $CO_2$ production (and their standard deviations) in mmol $g_{dw}^{-1}$ $h^{-1}$

| Test type[a] | Number | Mean $O_2$ | SD $O_2$ | Mean $CO_2$ | SD $CO_2$ |
|---|---|---|---|---|---|
| a | 7 | 0.153 | 0.079 | 0.152 | 0.045 |
| b | 4 | 0.232 | 0.048 | 0.072 | 0.116 |
| c | 4 | 0.166 | 0.023 | 0.222 | 0.007 |
| d | 3 | 0.154 | 0.056 | 0.240 | 0.017 |

[a] Test types are explained in Sect. 2.4.

**Table 2.** Summary of gas exchange data from stoppered culture tests using *A. annua*. Two sample t test results

| Comparison | $O_2$ usage | Equality prob.[a] | $CO_2$ prod. | Equality prob.[a] | Conclusions[b] |
|---|---|---|---|---|---|
| a vs. b | a < b | 0.073 | a > b | 0.28 | none |
| a vs. c | a < c | 0.71 | a < c | 0.007 | c more active |
| a vs. d | a < d | 0.99 | a < d | 0.003 | d more active |

[a] Equality probability is the likelihood that a difference at least as large as that observed would have occurred if the two samples being compared had come from the same population. A large value suggests the two samples are indistinguishable; a small value suggests a true difference.

[b] The conclusions in the end column are based on comparisons of both specific oxygen usage rate and specific carbon dioxide productivity. If either trait shows a statistically significant difference (at the 95% level), we conclude that there is an activity difference.

No significant difference was observed.

2 a vs. c (effect of using freshly inoculated culture vs. 7–8-day-old culture at the start of the test):

The older cultures had significantly higher $CO_2$ productivity. The $O_2$ usage rates were not significant different. The inoculum evidently included a significant amount of dead biomass. A 7–8-day-old culture would have the same amount of dead material as it had at the time of inoculation, but this dead material would make up a smaller fraction of the total. The higher $CO_2$ production per unit amount of biomass suggests that this is what occurred in this study. It is difficult to explain why this difference manifested itself only in $CO_2$ production and why $O_2$ usage was nearly unchanged.

3 a vs. d (effect of age of inoculum):

Cultures started with 10-day-old inoculum produced significantly more $CO_2$ than did those started with 21-day-old inoculum. Again, there was no difference in $O_2$ usage rates. This appears to suggest that much of the 21-day-old inoculum was dead, agreeing with the interpretation of the a vs. c test results (2) above. Partly as a result of this experiment, it was decided to subculture every 10 or 11 days (twice every 3 weeks).

Plots of headspace gas data are presented for the type 'a' tests, which were used as a basis of the comparisons that were analyzed statistically and listed above. Gas concentration plots for oxygen, carbon dioxide and ethylene are presented in Fig. 1.

Ethylene and carbon dioxide are produced by the plant cells in the culture and their respective headspace concentrations increase in the stoppered flasks. As expected, oxygen consumption by the cells results in reduction of the headspace composition. Specific $O_2$ consumption rate and biomass accumulation curves as a function of time are presented in Fig. 2. The biomass accumulated at a rate of approximately 0.05 $g_{dw}\ l^{-1}\ h^{-1}$; the average specific $O_2$ consumption rate calculated as 0.05 mmol $g_{dw}^{-1}\ h^{-1}$. The same data plotted as specific $O_2$ con-

sumption rate vs. $O_2$ concentration in Fig. 3 indicates apparent first-order behavior, which contrasts with the zero-order consumption of $O_2$ observed by other investigators [31–33, 50]. It also contrasts with the results from the gas feed tests presented below. A few conventional cultures were analyzed for gas headspace composition [74]. Our best estimates for the compositions early in the test are 16.9% $O_2$, 5.9% $CO_2$ and 1.7 ppm $C_2H_4$. The difficulty of sampling these cultures without admitting laboratory air makes the measurements uncertain. Also, these concentrations, unlike those from the gas feed cultures discussed below, changed with time, at least until diffusion through the foam-and-foil stoppers came to equilibrium with the chemical processes in the cells. Thus, even if we knew the *average* concentrations in these tests, maintenance of those

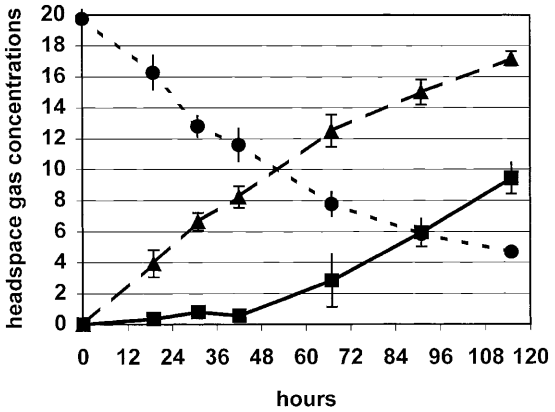



**Fig. 1.** Headspace concentrations of oxygen, carbon dioxide and ethylene from type 'a' cultures of *A. annua* as a function of culture age. Ethylene (■); carbon dioxide (▲); oxygen (●)

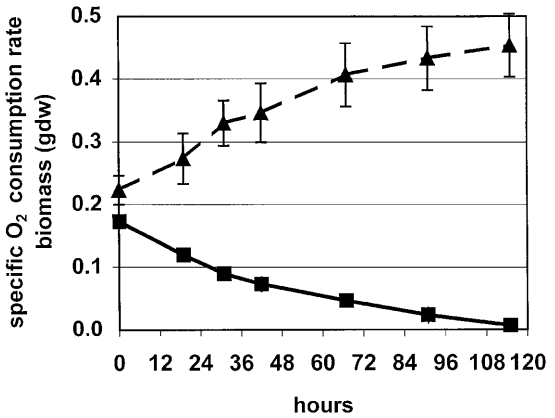



**Fig. 2.** Specific oxygen consumption rate (g $O_2$ $g_{dw}^{-1}$ $h^{-1}$) and biomass accumulation (gdw per culture flask) of *A. annua* from type 'a' cultures as a function of time. Consumption (■); biomass (▲)

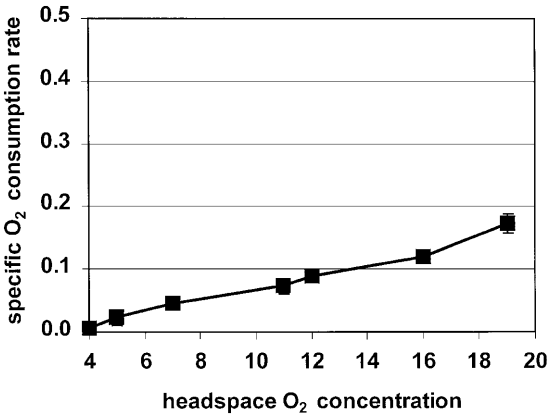



**Fig. 3.** Specific oxygen consumption rate (g $O_2$ $g_{dw}^{-1}$ $h^{-1}$) of *A. annua* from type 'a' cultures as a function of oxygen headspace concentration

concentrations continuously through a test might well lead to different culture behavior than that observed from conventional cultures.

The growth ratio of $final_{dw}$ to $initial_{dw}$ (FDW/IDW) and glucose concentration as functions of time are shown in Fig. 4. Since the growth ratio data became divergent and the glucose levels decreased to low levels after 10–11 days, it was decided to routinely subculture using 10- or 11-day-old inoculum (that is, two generations every 3 weeks). The medium for the cultures contains sucrose, which is hydrolyzed to glucose and fructose within a few days of initiating the culture. Glucose is consumed before fructose, so, even at the low levels of glucose shown in Fig. 4, the energy source is not the limiting nutrient in the flask cultures described here.

Results of a two-tailed t test comparing the FDW/IDW obtained from conventional cultures and the gas feed cultures that were run at the same time in-

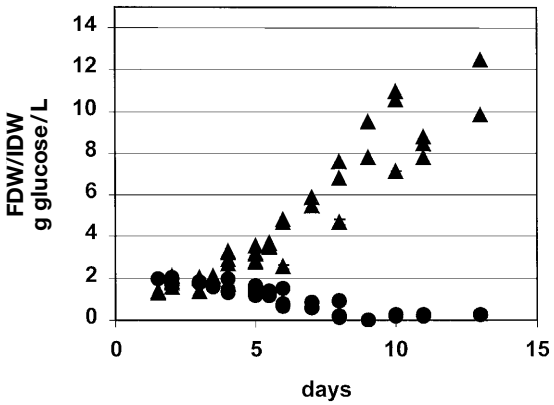



**Fig. 4.** Growth ratio (FDW/IDW) and final glucose concentration (g glucose $l^{-1}$) in cultures of *A. annua* type 'a' cultures as a function of time. Growth ratio (■); glucose (▲)

dicated a 6% probability (at the 94% confidence level) there would have been as much (or more) difference between the mean and fit if the populations *were* indistinguishable. We conclude, therefore, that growth in conventional cultures was significantly more rapid than would occur in a gas feed culture with the same average headspace composition, subject to the qualifications of the previous paragraph.

Gas-phase composition was one of the prime considerations in assessing and controlling productivity in the plant cell cultures of *A. annua* and *Taxus cuspidata* that we studied at Colorado State University. A gas mixing station was constructed, following plans published by Barmore and Wheaton [75], in order to develop full-factorial design experiments and provide 18 culture flasks with separate independent mixtures of $O_2$, $CO_2$ and $C_2H_4$ [28, 74]. The flow to each flask replaced the headspace at a rate of 40 ml $min^{-1}$ without bubbling into the 40 ml of medium in the 125-ml flasks. Using reduced oxygen levels (10% v/v), elevated carbon dioxide (0.5% v/v) and elevated ethylene (5 ppm), paclitaxel yield was improved in terms of reduced time before paclitaxel appeared in the culture medium and by increased paclitaxel accumulation in the medium [28].

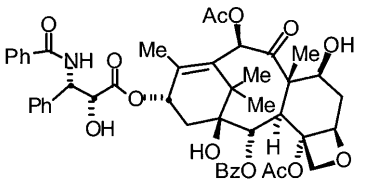
Table 3 is a representation of the dependence of culture growth and artemisinin production by *A. annua* on the concentrations of oxygen and carbon dioxide in the gas feed system. The FDW/IDW ratio increased with oxygen headspace gas concentration to about the ambient concentration (21% v/v) and then decreased at greater concentrations of oxygen. There appears to be no relationship between artemisinin production and oxygen concentration. However, the lower concentrations of carbon dioxide were found to promote the greater productivity during the 2-week period of the experiment and higher carbon dioxide levels appear inhibitory. Carbon dioxide is known to inhibit ethylene action [38], which may have been masked by the large variability of these results.

**Table 3.** Effect of headspace gas composition on artemisinin and growth of cultures in gas fed systems. Data represent averages and standard deviations of 5–7 replicate flasks, each supplied with the given oxygen and carbon dioxide composition and various ethylene concentrations. Because regression analysis did not indicate ethylene to have significance in determination of these parameters at the 90% confidence level, all ethylene concentrations were lumped in the given oxygen and carbon dioxide concentrations

| $O_2$ % (v/v) | $CO_2$ % (v/v) | FDW/IDW g/g | SD | Artemisinin µg/g IDW | SD |
|---|---|---|---|---|---|
| 9.1 | 3.0 | 2.9 | 1.6 | 14.3 | 9.7 |
| 10.8 | 12.8 | 6.2 | 5.4 | 0.0 | 0.0 |
| 16.1 | 7.2 | 11.6 | 9.5 | 6.6 | 7.5 |
| 23.0 | 6.7 | 15.6 | 15.9 | 0.0 | 0.0 |
| 32.5 | 1.1 | 14.4 | 9.9 | 24.9 | 5.9 |
| 47.9 | 2.8 | 3.0 | 0.7 | 10.0 | 8.2 |

## 3
## *Taxus cuspidata* and *T. canadensis* Production of Paclitaxel: Dependence on Dissolved Gas Concentrations

Paclitaxel is a plant-derived drug used in the treatment of breast, ovarian and lung cancers; clinical trials are underway for treatment of other cancers. The extraction and purification of paclitaxel was initially from Pacific yew trees *T. brevifolia* and shrubs and trees of other *Taxus* species: *T. baccata*, *T. cuspidata*, *T. sumatrana*, *T. chinensis*, *T. yunnanensis* and *T. hicksii*. Because the evergreen *Taxus brevifolia* grows slowly (roughly a foot of height and a half inch of trunk diameter per decade), other techniques were considered to produce the compound without destroying *T. brevifolia* trees. Bristol-Meyer Squibb is currently manufacturing paclitaxel using a semi-synthesis from 10-deacetylbaccatin III, which is isolated from needles of the Himalayan yew, *T. wallinchina*.




**Structure 2.** Paclitaxel **1**, taxol

The cell culture process was licensed in May 1995 by Bristol-Meyer Squibb, which in 1998 designated $25 million for development of an FDA-approved commercial process. Many academic and industrial research groups around the world are pursuing plant cell culture routes of production [76–86].

A significant contribution was made to the science of plant cell culture production of paclitaxel. Methyl jasmonate solutions that had been pipetted into cell suspension cultures caused transient increases in paclitaxel production [27]. This was the first report of modeling that indicated ethylene and methyl jasmonate may participate in "cross-talk" signal transduction in plants. Other papers have subsequently appeared which demonstrate enhancement of paclitaxel productivity by the application of methyl jasmonate to several *Taxus* species [87, 88].

The interdependence of methyl jasmonate with chitin- and chitosan-derived elicitors was studied using plant cell suspension cultures of *Taxus canadensis*. Induction of the biosynthesis of paclitaxel and other taxanes was enhanced when methyl jasmonate and elicitors were added 8 days after culture transfer compared to treatments in which only methyl jasmonate or only elicitor was added. The optimal elicitor concentration using *N*-acetylchitohexaose was 0.450 µM, but only in the presence of methyl jasmonate. Little, if any, induction of taxane formation occurred with the oligosaccharide alone. The optimal methyl jasmonate concentration was 200 µM using colloidal chitin or oligosaccharides of chitin and chitosan as elicitors.

Simple carbohydrates and lipids are proving important as signal induction mediators for regulation of plant growth and development. Fungal cell wall derived oligosaccharides are one group of the former. Methyl jasmonate (MJ) is a lipid-derived elicitor. Both classes of elicitors activate signal transduction pathways and regulate expression of genes for production of phytoalexins and other secondary metabolites. Paclitaxel and other taxanes, such as baccatin III, 10-deacetyltaxol and 10-deacetylbaccatin, are elicited by these materials in *Taxus canadensis* suspension cultures.

Fatty acid signaling in plants has been reviewed by Farmer [89]. Jasmonic acid arises in plants from $\alpha$-linolenic acid via the octadecanoic pathway by:

1 release of linolenic acid from membranes,
2 oxidation of linolenic acid by 13-lipooxygenase (13-LOX), a plastid system, and
3 conversion of the 13-hydroperoxide to jasmonic acid by allene oxide synthase (AOS), AO cyclase (AOC), a reductase and three rounds of $\beta$-oxidation, which are all constitutive in non-induced plants [90]. This rapid, but transient, synthesis of *cis*-jasmonic acid has been demonstrated in whole plants and in suspension cultures. Many plant species tested in cell suspension culture were elicited by exogenously supplied MJ with respect to the accumulation of secondary metabolites [91–93]. Addition of MJ initiates de novo transcription of genes, such as phenylalanine ammonia lyase and peroxidases, which are involved in some chemical defense mechanisms of plants and the synthesis of early intermediates of secondary products [92], including the *N*-benzoyl-3-phenylisoserine side chain of paclitaxel. MJ induced rosmarinic acid biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures [94] and shikonin, the red naphthoquinone pigments of the root, and dihydroeihenofuran, an abnormal benzofuran metabolite [94]. Wounding-induced anthocyanin and flavonoid synthesis in petunia was enhanced by MJ [95]. Cooperative stimulation by ethylene and MJ of paclitaxel formation in *T. cuspidata* [27] and *T. canadensis* [29] has been reported.

### 3.1
### Optimization of Headspace Composition on Production of Paclitaxel

The use of a statistical regression model for optimization of headspace composition on production of cell mass in the *A. annua* system is presented in section 4 of this chapter. A similar exercise for optimization of headspace composition on paclitaxel productivity was conducted. The effect of controlled headspace gas composition for suspension cultures of *T. cuspidata* was analyzed from data of Mirjalili and Linden [28] using the statistical program SAS to identify the optimum gas combination and the interaction of each parameter. A linear regression technique was used to fit the data to quadratic equations containing variables in the system. The sign and magnitude of the coefficients of each factor allowed comparison of important parameters. The optimization was obtained from the derivative of the quadratic Eq. (17):

$$\text{Paclitaxel productivity (mg l}^{-1}\text{ day}^{-1}) = -2.073 + 0.307[O_2] + 0.075\,[\log(CO_2)] + 0.366\,[\log(C_2H_4{+}1)] - 0.009\,[(O_2)^2] + 0.001[O_2 \times \log(CO_2)] - 0.120\,[(\log(CO_2))^2] - 0.012\,[\log(C_2H_4{+}1) \times O_2] - 0.076\,[\log(C_2H_4 + 1) \times \log(CO_2)] - 0.091[(\log(C_2H_4 + 1))^2] \quad (17)$$

At the maximum point, ethylene, oxygen and carbon dioxide concentrations are 6.29 ppm, 17.00 % (v/v) and 1.35 % (v/v), respectively, with 0.70 mg $l^{-1}$ $day^{-1}$ paclitaxel productivity. The statistical analysis shows that the terms $\log(C_2H_4{+}1)$, $(O_2)$, $(O_2)^2$, $(\log CO_2)^2$, $[\log(C_2H_4 + 1) \times (O_2)]$, and $[(\log(C_2H_4 + 1))]^2$ have effects at the 95 % significance level on paclitaxel production. The effects of $\log(C_2H_4 + 1)$ and $(O_2)$ are positive, while all the other effects are negative. The effects of gas mixture composition on paclitaxel productivity are visualized in the response surface diagrams in Fig. 5.

### 3.2
### Application of Volatile Methyl Jasmonate to Headspace Flow

Elicitors evoke responses in whole plants by a variety of routes. Touch produces dramatic responses to a number of plants such as *Mimosa*, and systemic responses to an elicitation encounter on one part of a plant become expressed on distal plant parts within a short period of time. Elicitation events on one plant in a chamber which cause the biosynthesis of methyl jasmonate (MJ) as a primary response to the elicitation and a secondary response as a effect of the methyl jasmonate have been shown to produce the secondary response on other plants in the chamber that are not elicited. The volatility of methyl jasmonate is

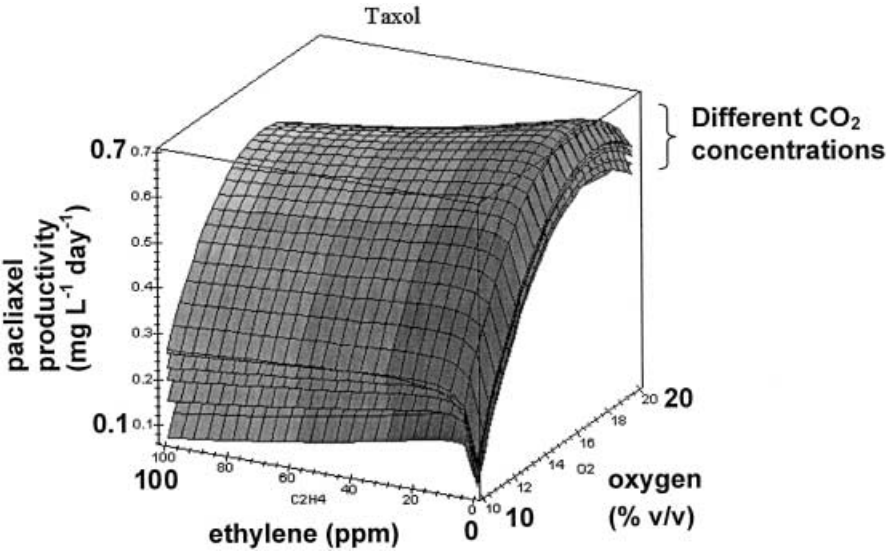



**Fig. 5.** Dependence of paclitaxel production on headspace gas concentrations of ethylene, carbon dioxide and oxygen according to the linear regression model

thought to carry the signal from the elicited plant to the surrounding unelicited plants.

Elicitation of plant cell suspension cultures is normally carried out by addition of a solution of the elicitor. Indeed, for investigators without the means for mixing gases and supplying the mixture to the plant cell culture, application of the plant hormone ethylene is normally conducted by addition of a dilute ethephon solution to the plant suspension culture. As noted above, ethephon breaks down to ethylene, HCl and phosphate when exposed to water. The concentration of ethylene dissolved in the culture growth medium, calculated using Henry's Law, in equilibrium with headspace containing 10 ppm ethylene is 0.067 µM. High concentrations of dissolved ethylene inhibit paclitaxel accumulation, as demonstrated using 25 and 50 ppm headspace ethylene levels in cultures that were not also elicited with MJ [27].

Similarly, MJ is normally dissolved in ethanol and small aliquots are added to the plant cell suspension culture to deliver the appropriate concentration, for example 10 or 100 µM [27]. In experiments using 10 µM MJ, paclitaxel formation is stimulated in the presence of 5 ppm headspace ethylene in equilibrium with the cell culture. However, 100 µM MJ is required to produce paclitaxel in flasks exposed to continuous flowing gas mixtures containing 10 ppm ethylene. Paclitaxel productivity at 5 ppm $C_2H_4$ and 10 µM MJ was 19-fold higher than the basal level; at 5 ppm $C_2H_4$ and 0 µM MJ (with ethanol), the increase was 3-fold; with 0 ppm $C_2H_4$ and 10 µM MJ, the increase was 15-fold; with 5 ppm $C_2H_4$ and 0 µM MJ (no ethanol), this increase was also 15-fold. The observed differences may depend on ethylene biosynthesis by the cultures; elicited cultures may have accumulated levels of ethylene that became inhibitory to paclitaxel formation. These results are demonstrated in Fig. 6.

Experiments were conducted in which the gas mixture flowing to the culture flask contained MJ as well as ethylene [in the optimum combination with 17% (v/v) $O_2$ and 1.5% (v/v) $CO_2$]. The results given in Fig. 7 demonstrate clearly that ethylene and MJ co-mediate the induction of paclitaxel biosynthesis. When MJ was supplied by passing an air stream over concentrated MJ in a flask, no product formation was noted. When the optimal gas mixture was applied to the headspace of the culture without first passing over the pure MJ in a flask, the level of paclitaxel accumulation was about 20% of that found in the culture to which both MJ and $C_2H_4$ had been applied by means of the vapor phase. The 8-day period of time before induction may represent the time required for the critical concentration of MJ to dissolve in the culture medium under the flow (40 ml total gas mixture $min^{-1}$) and temperature (21 °C) conditions of the experiment.

The effect is not transient, as for the case of injection of the MJ solution into the culture (Fig. 6), where the induction of paclitaxel occurred within 51 h after elicitation and began declining again within 24 h. The vapor pressure and Henry's constant for MJ were unknown, at least to these investigators; until some of the physical characteristics of MJ are known, there is no means of evaluating the MJ evaporation and dissolution rates.

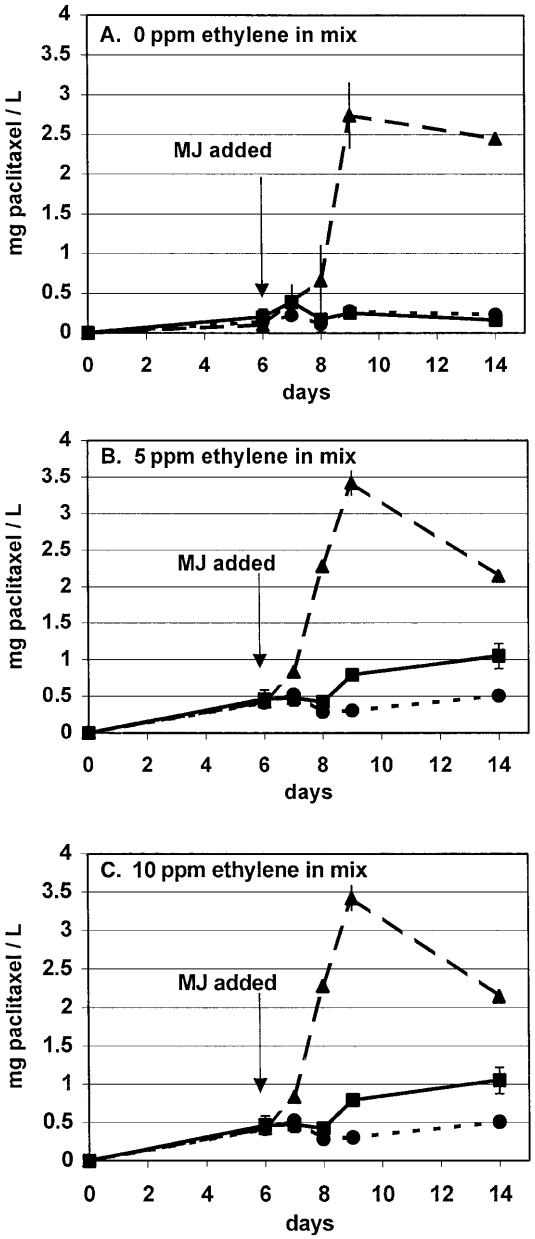



**Fig. 6.** Ethylene and methyl jasmonate (MJ) interaction when MJ is supplied as ethanolic solution to the suspension cell cultures of *T. cuspidata*. All cultures were exposed to 10% (v/v) oxygen, 0.5% (v/v) carbon dioxide and designated headspace concentrations of ethylene. **A** 0 ppm; **B** 5 ppm; **C** 10 ppm. 0 µM MJ (●); 10 µM MJ (■); 100 µM MJ (▲). Reprinted with the permission of the American Chemical Society and the American Institute of Chemical Engineers [27]

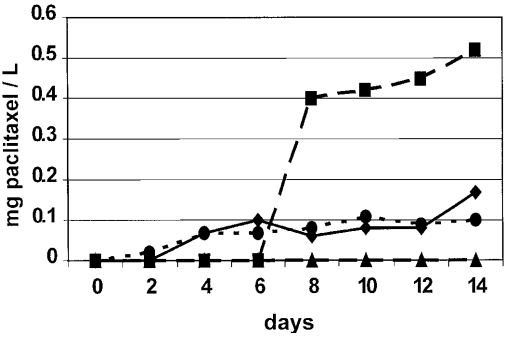



**Fig. 7.** Ethylene and methyl jasmonate (MJ) interaction when MJ is supplied in gas phase to the suspension cell cultures of *T. cuspidata*. MJ in optimal gas mixture of 17% (v/v) oxygen, 1.5% (v/v) carbon dioxide and 5 ppm ethylene starting on day 1 of the experiment (■); MJ in optimal gas mixture starting on day 8 of the experiment (◆); MJ in air (▲); optimal gas mixture without MJ (●)

### 3.3
### Formation of Ethylene and Methyl Jasmonate Upon Elicitation of Plant Cell Cultures

Thirty-six plant species tested in cell suspension culture could be elicited by exogenously supplied methyl jasmonate to accumulate secondary metabolites by a factor of 9 to 30 over the control values. Induction by MJ was not specific to any one type of secondary metabolite but rather general to a wide spectrum of low molecular weight substances ranging from flavonoids, guaianolides and anthraquinones to various classes of alkaloids [96]. Endogenous jasmonic acid and its methyl ester (MJ) accumulate rapidly and transiently after treatment of plant cell suspension cultures of *Rauvolfia canescens* and *Eschscholtzia californica* with a yeast elicitor [97].

Two types of oligosaccharides, both potentially derived from the chitin cell walls of pathogenic fungi, act as potent elicitors in suspension-cultured plant cells. The first of these, *N*-acetylchitooligosaccharides, induce phytoalexin (momilactones and oryzalexins) formation in rice cells even at nM ranges [98]. The affinity constant between 1 nM and 10 nM corresponded well to the concentration with which this class of oligosaccharide induces responses in the cultured cells. Inhibition studies with various oligosaccharides also showed the binding specificity of this site corresponded well with that observed for the cultured cells, which support the possibility that this binding site represents a true, functional receptor for this elicitor [4].

Secondly, the deacetylated form of chitin, chitosan, does not induce phytoalexin formation in the rice system but is active in other plant culture systems [99]. Glucan elicitors induce phytoalexins in legumes (soybean, chickpea, bean, alfalfa, pea) and solanaceous sp. (potato, sweet pepper) [100]. However, anthraquinone biosynthesis was stimulated in *Morinda citrifolia* by both chitin and chitosan. The degree of acetylation was found to be important in inducing defense responses. During the first few days of incubation after adding elicitor,

production of chitinase increased and then declined when anthraquinone biosynthetic enzymes became active [93].

Co-mediation of oligosaccharides and MJ has been demonstrated in the rice system in the induction of phytoalexins [100]. Exogenously applied MJ to elicited cells increased production of momilactone A to levels higher than those elicited with *N*-acetylchitoheptaose alone. In suspension-cultured cells of parsley the influence of MJ on elicitation using cell walls of *Phytophtora megasperma* (Pmg elicitor) and chitosan was demonstrated [101]. These results suggested MJ conditioned the parsley suspension cells in a time-dependent manner to become more responsive to elicitation.

Studies of ethylene production by cell cultures were conducted under the following conditions:

A control with appropriate replacements of medium in which elicitors are dissolved;
B 100 µM MJ;
C 28 µM (37 mg $l^{-1}$) *N*-acetylchitohexaose;
D 100 µM MJ+28 µM *N*-acetylchitohexaose.

Data shown in Fig. 8 indicate rapid accumulation of ethylene in each of the flasks during the first 4 days following elicitation. MJ causes the greatest accumulation of ethylene; *N*-acetylchitohexaose appears to inhibit ethylene biosynthesis both with and without MJ in that accumulation is less than that in the control.

These results are comparable in terms of relative ethylene concentrations 4 days after elicitation to similarly conducted experiments in which the cell cultures were not stoppered. The accumulation was 1000-fold less (and at the limit

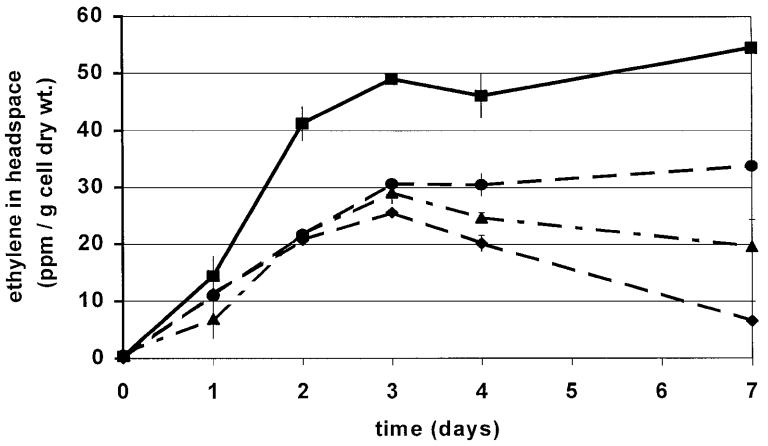



**Fig. 8.** Seven-day ethylene production kinetics by 40 ml *Taxus canadensis* cultures, which were in 125-ml Erlenmeyer flasks closed with serum caps, following elicitation as follows: (■) methyl jasmonate (100 µM); (●) control; (▲) combination of *N*-acetylchitohexaose (37 mg $l^{-1}$) and methyl jasmonate (100 µM); (◆) *N*-acetylchitohexaose (37 mg $l^{-1}$). Reprinted with the permission of Elsevier Science, Ireland Ltd. [103]

of detection) because ethylene diffused through the silicone closures (data not shown). The results from the replicated stoppered flasks are shown, because the reproducibility of the experiment is better. The concentration of ethylene in the medium in equilibrium with headspace containing 10 ppm, calculated using Henry's Law, is 0.067 µM. In the unstoppered flasks, from which measured ethylene concentrations were three orders of magnitude less, dissolved ethylene concentrations are in the order of 0.1 nM at equilibrium. In a system for studying ethylene-induced chitinase, Boller et al. found exogenously supplied ethylene at 1 ppm (presumed headspace concentration) was sufficient for half-maximal induction of chitinase activity and enhancement of the endogenous ethylene formation [102].

The greatest realization from this study has been learning the importance of ethylene in the elicitation process. Ethylene biosynthesis by this particular system in response to MJ or oligosaccharides is demonstrated. While MJ stimulates ethylene formation, the chitosan- and chitin-oligosaccharides used in this study inhibit ethylene biosynthesis by the plant cell culture. Depending on the physiological status of the culture at the time of elicitation, the ethylene concentration may effectively be great enough at the site of action in the cells to promote the MJ/ethylene co-mediation [103]. Additional experimental detail is available in the respective thesis and dissertations [104 – 107].

## 4 Conclusions

Several conclusions are presented here. Suspension cultures of *A. annua* grown by our procedure exhibit logarithmic growth with no measurable lag phase, for 8 to 10 days. The specific growth rate averaged 0.227 inverse days (data not shown). This estimate may or may not have been skewed by procedural problems. If the ratio of intracellular dissolved solids to insoluble cell material was unchanged with culture age, then the specific growth rate as calculated is correct. If, however, the ratio increases with culture age, then we have underestimated the growth rate (the opposite is true if that ratio decreases).

A very simple unstructured material balance model of suspension culture growth was developed. Data for cell mass and extracellular glucose remaining at culture harvest was fitted to the model to obtain model parameters. According to those values, 94% of the glucose consumption in our cultures was growth associated. Thus, only 6% was non-growth-associated "cell maintenance" [108]. This estimate is subject to uncertainty due to both the model assumptions and errors in the growth data as described above.

The stoppered culture tests generated initial specific rates of $O_2$ consumption and $CO_2$ production by suspended *A. annua* cells grown under various conditions. The apparent specific productivities of ethylene were disappointingly inconsistent and are thus not reported. The "initial" specific rates were those rates occurring shortly after the cultures were stoppered, before the concentrations of gases changed drastically.

The most significant effect of culture conditions upon those initial rates was the effect of inoculum age. Cultures started with 10-day-old inoculum showed

significantly greater $CO_2$ productivity than did cultures started with 21-day-old inoculum. Partly because of this, it was decided to routinely subculture using 10- or 11-day-old inoculum (that is, two generations every 3 weeks).

Initial $CO_2$ productivity determined in the stoppered culture tests was used in conjunction with growth rate and glucose consumption model parameters to perform a material balance on carbon. The productivity, which was determined for cultures started with 10-day-old inoculum, was used because the cultures used to generate growth rate data were started with 10- or 11-day-old inoculum. It was not possible to obtain all the data for this material balance from a single culture. Therefore, a "typical" culture was hypothesized that possessed the growth and carbon usage behavior of the growth test cultures and the $CO_2$ productivity as measured in the gas feed tests. The fate of the carbon in the glucose consumed would be as follows: 49% to biomass, 31% to $CO_2$ and 20% to extracellular solutes (calculated by difference). Some or all of the latter quantity was probably intracellular dissolved organic compounds released into the medium by vigorous centrifugation.

Data from the stoppered culture tests was also used to determine the effect of declining $O_2$ concentration on the rate of metabolism of the cells. The specific $O_2$ consumption rate was determined to depend linearly upon the $O_2$ concentration throughout the range of headspace concentrations measured (0–31%). This seems to contradict the experience of other researchers [31–33, 50], who report saturation kinetics in plant cell cultures whenever $O_2$ concentration exceeded 4–5% (gas phase). In the stoppered culture tests, at the same time $O_2$ concentration declined, $CO_2$ and $C_2H_4$ concentrations increased. Other unidentified compounds may also have been produced. The declining rate of metabolism observed may have been caused by conditions other than declining oxygen.

A system of tubing and manifolds was constructed to test the effect of dissolved gas concentrations on culture behavior. Because cellular metabolism in cultured plant cells is relatively slow (compared to that of cultured microbes), gas equilibrium is established between the culture headspace and the medium. Using the gas feed system, we studied the effect of varying $O_2$, $CO_2$ and ethylene concentrations on culture growth. According to a multivariable regression, the ratio (FDW/IDW) of final to initial dry weight of a culture is given by the following function of the gas concentrations:

$$\begin{aligned}\mathrm{FDW/IDW} = {} & 11.2 + 0.0361 \cdot O_2 \cdot CO_2 + 0.0534 \cdot CO_2 \cdot \\ & 2 - 1.62 \cdot CO_2 + 0.0355 \cdot C_2H_4 - 0.189 \cdot O_2 \end{aligned} \quad (18)$$

Here the terms are in order of statistical significance. It is reasonable to suspect that if $O_2$ consumption were a first-order function of its concentration, growth would be likewise. The regression equation above would be dominated by the first-order $O_2$ term. In fact, in Eq. (18), growth is a relatively weak function of $O_2$. This suggests that metabolic activity was not a first-order function of $O_2$ concentration in the concentration range we investigated.

Artemisinin was produced by some *A. annua* cultures, as were three other electrochemically reducible compounds, based on the type of HPLC detector used for analysis of the peroxide-bridged, functionally active materials in the

**Table 4.** Volumetric productivities from several microbial and plant secondary metabolite production systems

| Product | Organism | Vol. Prod.[a] | Reference | Culture type |
|---|---|---|---|---|
| Penicillin[b] | *Penicillium crysogenum* | 3.2 | 24 | submerged cultures |
| Sanguinarine[c] | *Papaver somniferum* | 0.14 | 23 | suspension cultures |
| Shikonin[c] | *Lithospernum erythrorhizon* | 0.1 | 20 | suspension cultures |
| Berberine[c] | *Thalictrum rugosum* | 0.004 | 65 | suspension cultures |
| Berberine[c,d] | *Thalictrum rugosum* | 0.12 | 42 | immobilized cell cultures |
| Artemisinin[e] | *Artemisia annua* | 0.003 | 70 | plant leaves |
| Artemisinin[c] | *Artemisia annua* | 0.000007 | gas feed cultures this work | |
| Paclitaxel[c] | *Taxus canadensis* | 0.022 | 86 | suspension cultures |
| Paclitaxel[c] | *Taxus canadensis* | 0.16 | 30 | semicontinuous suspension cultures with total recycle |

[a] Volumetric productivity is in $g_{product}\, l^{-1}\, day^{-1}$.
[b] The penicillin productivity is reported in the reference as that achieved in the commercial process.
[c] These compounds were produced by cultured plant cells. The volume of the culture (rather than the vessel volume) was used in the calculation.
[d] Volumetric productivity is calculated from available cited data.
[e] The "productivity" of *A. annua* plant leaves is the quantity of artemisinin divided by the growth time divided by volume of leaf material (assuming a density of 1 kg $l^{-1}$).

culture medium. For each of these compounds, the quantity produced per culture was regressed against the headspace gas concentrations. Unfortunately, a great deal of random variation occurred for each compound. The random uncertainty turned out to be greater than the variation accounted for by headspace composition. For most of those compounds, the most statistically significant variable was the product of the $O_2$ and $CO_2$ concentrations. In no case did we see a significant effect of ethylene concentration on productivity. A study involving more replicates and a greater range of ethylene concentrations might possibly demonstate such a dependence.

The average volumetric productivity of artemisinin, the amount of product generated divided by culture volume and by culture age at harvest, was calculated for the most successful gas feed cultures. Those were the cultures fed 32.5% $O_2$ and 1.14% $CO_2$. In Table 4 this volumetric productivity is compared with productivities in a fungal fermentation, several phytoproduction processes reported in the literature and greenhouse-grown *A. annua* plants. Clearly, the productivity achieved in this work was extremely low, even in comparison to other phytoproduction processes. Other investigators of *A. annua* report little success producing artemisinin from cell cultures [70].

On the other hand, the impact of ethylene in the composition of headspace gases fed to *Taxus* sp. cultures was very evident. The positive involvement of ethylene was also noted when cultures were elicited with both dissolved and volatilized methyl jasmonate and with chitin- and chitosan-derived oligosaccharides. The effect of the latter compounds on biosynthesis of ethylene by the plant cell cultures brings us to a better understanding of the interdependence of elicitor and hormone concentrations and of "cross-talk" signal transduction in plants.

Plant hormones rarely act alone; hormones interact to produce a final effect. According to Gaspar et al., "Some responses of plants to auxins may be caused by increased ethylene synthesis in response to auxin treatment. At high ethylene concentrations, microtubule and microfibril orientation are altered, which results in decreased cell elongation and increased cell expansion. The role of ethylene is hard to understand because it effects vary with developmental stage and because low concentrations can promote (or sometimes inhibit) a process, whereas higher levels have the opposite effect" [22].

Methyl jasmonate and ethylene act as co-mediators of cellular responses in many plant systems [109, 110]. We hypothesize that specific biosynthetic steps for taxol production in plant cell culture are regulated by ethylene and MJ interaction, which is based on our induction modeling and observed allosteric effects between MJ and ethylene [27, 29]. Our literature review has uncovered nothing about MJ receptor or MJ binding constants. On the other hand, binding sites for ethylene on intercellular membranes from many plant genera have been established for years [111, 112] and, recently, the transmembrane ethylene receptor protein, ETR1, has been localized using fluorescent-labeling techniques to the endoplasmic reticulum [113]. ETR1 and other ethylene-binding proteins, ERS1, EIN4, ETR2 and ERS2, have been characterized to contain copper ions on sulfhydryl groups buried within the membrane and to contain variability with respect to the sensor and transmitter functions of two component regulators [114–116]. Whereas complete motifs are absent in EIN4, ETR2 and ERS2, ETR1 and ERS1 contain signature motifs found in histidine protein kinases and have been shown to interact with the gene product of CTR1, a Raf protein kinase homologue that is a negative regulator of ethylene signal transduction [115].

The initial steps of the signal pathway for ethylene are at least known to have similarity to two-component regulators of eukaryotes. Each component contains a conserved domain and a variable domain. Most sensor proteins consist of a variable amino-terminal domain (typically located in the periplasmic space flanked by two transmembrane domains) and a conserved carboxyl-terminal histidine kinase domain located in the cytoplasm. Signal perception on the *N*-terminal domain results in autokinase activity by the histidine kinase domain. The phosphate of the histidine is transferred to a certain aspartate residue within the conserved amino-terminal domain of the cognate cytoplasmic response regulator [116].

In attempting to model the MJ/ethylene system, we view a two-step process: MJ absorption in the membrane is directly related to MJ concentration, but its interaction with one or more of the ethylene binding proteins is effective only at higher concentrations. Hence, at low to high MJ concentrations, the modulat-

ed ethylene binding initiates signaling steps toward induction of enzymes that lead to synthesis of paclitaxel. The amount of ethylene binding is dependent on the MJ concentration and sigmoidal plots of paclitaxel are found [29]. This allosteric behavior indicates direct modulation of ethylene binding by MJ. At medium to high MJ concentrations, the modulation site is saturated, and no greater productivities are seen. Other MJ derivatives or lipid factors may also be involved [117–119].

Xu et al. hypothesize that the binding of ethylene to its receptors on the plasma membrane might sensitize MJ receptors on the membrane [110]. Our view is just the opposite: MJ may act as an "ethylene sensitivity factor" in modulation of ethylene binding to ETR1, which initiates the intracellular signal cascade that results, ultimately, in the accumulation of secondary compounds. The mode of action may involve non-specific dissolution of the lipid-derived MJ in the membranes, decrease in the order of regions of the phospholipid bilayer, and direct alteration of ethylene binding to ETR1. Phosphorylation of the aspartate activates or inhibits the variable C-terminal domain of the response regulator, which in some cases is a direct transcription activator [120].

## References


1. Staba EJ (ed) (1980) Plant tissue culture as a source of biochemicals. CRC Press, Boca Raton, Florida
2. Buitelaar RM, Tramper J (1992) J Biotechnol 23:111
3. Curtin ME (1983) Bio/technology 1:649
4. Dörnenburg H, Knorr D (1995) Enzyme Microb Technol 17:674
5. Sahai O, Knuth M (1985) Biotechnol Prog 1:1
6. Taticek RA, Moo-Young M, Legge RL (1991) Plant Cell Tissue Organ Culture 24:139
7. Walton NJ (1992) Chem Br 28:525
8. Morris P, Scragg AH, Stafford A, Fowler MW (eds) (1986) Secondary metabolism in plant cell cultures. Cambridge University Press, Cambridge
9. Payne GF, Bringi V, Prince CL, Shuler ML (1992) Plant cell and tissue culture in liquid systems. Hanser Publishers, Munich
10. Routian JB, Nickell LG (1956) US Patent 2 747 334
11. Bringi V, Kadkade PG, Prince CL, Schubmehl BF, Kane EJ, Roach B (1995) US Patent 5 407 816
12. Sanrou T, Mayumi Y, Kazuki I, Tae O, Masahiro A, Minoru K (1998) JP Patent 10 042 888A
13. Choi HK, Adams TL, Stahlhut RW, Kim SI, Yun JH, Song BK, Kim JH, Song JS, Hong SS, Lee HS (1999) US Patent 5 871 979
14. Flores HE, Vivanco JM, Loyola-Vargas VM (1999) Trends Plant Sci 4:220
15. Fett Neto AG (ed) (1996) Plant cell culture production of secondary metabolites. CRC Press, Boca Raton, p 139
16. Nakagawa K, Fukui H, Tabata M (1986) Plant Cell Reports 5:69
17. Obaata-Sasamoto H, Komamine A (1982) Suppression mechanism of DOPA accumulation in *Stizolobium* callus. In: Plant tissue culture 1982. Japanese Association for Plant Tissue Culture, Tokyo, p 345
18. Hashimoto T, Yamada Y (1986) Plant Physiol 81:619
19. Whitaker RJ, Hashimoto T (1986) Production of secondary metabolites. In Evans DA, Sharp WR, Ammirato PV (eds) Handbook of plant tissue culture, vol 4. Macmillan Pub Co, New York, p 264
20. Deno H, Suga C, Morimoto T, Fujita Y (1987) Plant Cell Rep 6:197

21. Constabel F (1990) Planta Med 56:421
22. Gaspar T, Kevers C, Penel C, Greppin H, Reid DM, Thorpe TA (1996) In Vitro Cell Devel Biol Plant 32:272
23. Roberts SC, Shuler ML (1997) Curr Opin Biotechnol 8:154
24. Crueger W, Crueger A (1984) Biotechnology: a textbook of industrial microbiology. Science Tech, Madison, p 206
25. Haigh JR, Linden JC (1989) Plant Cell Rep 8:475
26. Haigh JR, Linden JC (1991) Gen Eng Biotechnol 11:23
27. Mirjalili N, Linden JC (1996) Biotechnol Prog 12:110
28. Mirjalili N, Linden JC (1995) Biotechnol Bioeng 48:123
29. Phisalaphong M, Linden JC (1999) Ethylene and methyl jasmonate interaction and binding models for elicited biosynthetic steps of paclitaxel in suspension cultures of *Taxus canadensis*. In Kanellis AK, Chang C, Klee H, Bleecker AB, Pech JC, Grierson D (eds.) Biology and biotechnology of the plant hormone ethylene. Kluwer Academic Publishers, Dordrecht, p 85
30. Phisalaphong M, Linden JC (1999) Biotechnol Prog 15:1072
31. Hulst AC, Tramper J, Brodelius P, Eijkenboom LJC, Luyben KCAM (1985) J Chem Tech Biotechnol 35B:198
32. Hallsby GA (1984) MS thesis, Cornell University
33. Hallsby GA (1986) PhD dissertation, Cornell University
34. LaRue TAG, Gamborg OL (1971) Plant Physiol 48:394
35. Thomas DDS, Murashige T (1979) In Vitro 15:654
36. Zobel RW (1987) Environ Exp Bot 27:223
37. Fujiwara K, Kozai T, Watanabe I (1987) J Agr Met 43:21
38. Salisbury FB, Ross CW (1992) Plant Physiology, 4th edn. Wadsworth Publishing Co., Belmont, CA
39. Beyer EM, Morgan PW, Yang SF (1984) Ethylene. In: Wilkins MB (ed) Advanced plant physiology. Pitman Press, Bath, UK
40. Lieberman M, Wang SY, Owens LD (1979) Plant Physiol 63:811
41. Shuler ML, Sahai OP, Hallsby GA (1983) Ann NY Acad Sci 413:373
42. Facchini PJ, DiCosmo F (1991) Biotechnol Bioeng 37:397
43. Hsiao TY, Bacani FT, Carvalho EB, Curtis WR (1999) Biotechnol Prog 15:114
44. Rijhwani SK, Shanks JV (1998) Biotechnol Prog 14:442
45. Williams GRC, Doran PM (2000) Biotechnol Prog 16:391
46. McKelvey SA, Gehrig JA, Hollar KA, Curtis WR (1993) Biotechnol Prog 9:317
47. Perry RH, Chilton CH (1973) Chemical engineers handbook, 5th edn. McGraw-Hill, New York, pp 3–98
48. Weast RC (1985) CRC handbook of chemistry and physics, 65th edn. CRC Press Inc, Boca Raton, FL, p F-115
49. Hill CG (1977) An introduction to chemical engineering kinetics and reactor design, John Wiley & Sons, New York, p 451
50. Kobayashi Y, Fukui H, Tabata M (1989) Plant Cell Rep 8:255
51. Tate JL, Payne GF (1991) Plant Cell Rep 10:22
52. Su WW, Humphrey AE (1991) Biotechnol Lett 13:889
53. Smith JM, Davison SW, Payne GF (1990) Biotechnol Bioeng 35:1088
54. Fowler MW (1983) Commercial applications and economic aspects of mass plant-cell culture. In: Mantel SH, Smith H (eds) Plant biotechnology. Cambridge University Press, Cambridge, UK, p 3
55. Maurel B, Pareilleux A (1985) Biotechnol Lett 7:313
56. Stuhlfauth T, Klug K, Fock HP (1987) Phytochemistry 26:2735
57. Kim DI, Pedersen H, Chin CK (1991) Biotechnol Bioeng 38:331
58. Smart NJ, Fowler MW (1981) Biotechnol Lett 3:171
59. Abeles FB (1973) Ethylene in plant biology. Academic Press, New York, p 228
60. Freytag AH, Linden JC (1975) La Sucrerie Belge 94:429
61. Bagratishvili DG, Zaprometov MN (1988) Soobshch Akad Nauk Gruz SSR 131:385

62. Songstad KL, Giles J, Park D, Novakovski D, Epp L, Friasen L, Roewer I (1989) Plant Cell Rep 8:463
63. Kobayashi Y, Hara M, Fukui H, Tabata M (1991) Phytochemistry 30:3605
64. Kim DI, Pedersen H, Chin CK (1991) J Biotechnol 21:201
65. Cho GH, Kim DI, Pedersen H, Chin CK (1988) Biotechnol Prog 4:184
66. Luo XD, Shen CC (1987) Med Res Rev 7:29
67. Klayman DL (1985) Science 228:1049
68. Park JM, Hu WS, Staba EJ (1989) Biotechnol Bioeng 34:1209
69. Tawfiq NK, Anderson LA, Roberts MF, Phillipson MF, Bray DH, Warhurst DC (1989) Plant Cell Rep 8:425
70. Martinez BC, Staba EJ (1988) Adv Cell Culture 6:69
71. Bailey JE, Ollis DF (1986) Biochemical engineering fundamentals, 2nd edn. McGraw-Hill Book Co, New York, p285
72. Yegneswaran PK, Gray MR, Thompson BG (1990) Biotechnol Bioeng 36:92
73. O'Connor DJ, Dobbins W (1966) J Sanit Div Proc ASCE 82:SA6
74. Haigh JR (1993) PhD dissertation, Colorado State University
75. Barmore CR, Wheaton TA (1978) HortScience 13:169
76. Choi MS, Kwak SS, Liu JR, Park YG, Lee MK, An NH (1995) Planta Med 61:264
77. Gan FY, Peng LP, Zheng GZ (1996) Acta Bot Yunn 18:451
78. Griffin J, Hook I (1996) Planta Med 62:370
79. Hirasuna TJ, Pestchanker LJ, Srinivasan V, Shuler ML (1996) Plant Cell Tissue Organ Culture 44:95
80. Hoffman AM, Voelker CCJ, Franzen AT, Shiotani KS, Sandhu JS (1996) Phytochemistry 43:95
81. Kitagawa I, Mahmud T, Kobayashi M, Roemantyo J, Shibuya H (1995) Chem Pharm Bull 43:365
82. Liu DI, Zhang GY, Wang X, Hu ZB (1997) J Plant Resour Environ 6:48
83. Xiang W, Zhang HJ, Ruan DC, Sun HD (1997) J Plant Resour Environ 6:56
84. Zhiri A, Jaziri M, Guo Y, Vanhaelen Fastre R, Vanhaelen M, Homes J, Yoshimatsu K, Shimomura K (1995) Biol Chem Hoppe-Seyler 376:586
85. Mei X, Lu M, Yu L, Hu D (1996) Med Chem Res 6:256
86. Chattopadhyay SK, Kulshrestha M, Saha GC, Sharma RP, Jain SP, Kumar S (1997) J Med Arom Plant Sci 19:17
87. Ketchum REB, Gibson DM, Croteau R, Shuler ML (1999) Biotechnol Bioeng 62:97
88. Yukimune Y, Tabata H, Higashi Y, Hara Y (1996) Nature Biotechnol 14:1129
89. Farmer EE (1994) Plant Mol Biol 26:1423
90. Dammann C, Rojo E, Sanchez-Serrano JJ (1997) Plant J 11:773
91. Blechert S, Brodschelm W, Hoelder S, Kammerer L, Kutchan TM, Mueller MJ, Xia ZQ, Zenk MH (1995) Proc Nat Acad Sci USA 92:4099
92. Gundlach H, Mueller MJ, Kutchan TM, Zenk MH (1992) Proc Nat Acad Sci USA 89:2389
93. Kauss H, Krause K, Jeblick W (1992) Biochem Biophys Res Comm 189:304
94. Yazaki K, Takeda K, Tabata M (1997) Plant Cell Physiol 38:776
95. Tamari G, Borochov A, Atzorn R, Weiss D (1995) Physiol Plant 94:45
96. Kutchan TM, Dittrich H, Bracher D, Zenk MH (1991) Tetrahedron 47:5954
97. Yamada A, Shibuya N, Kodama O, Akatsuka T (1993) Biosci Biotechnol Biochem 57:405
98. Ito Y, Kaku H, Shibuya N (1997) Plant J 12:347
99. Cote F, Hahn MG (1994) Plant Mol Biol 26:1379
100. Nojiri H, Sugimori M, Yamane H, Nishimura Y, Yamada A, Shibuya N, Kodama O, Murofushi N, Omori T (1996) Plant Physiol 110:387
101. Kauss H, Jeblick W, Ziegler J, Krabler W (1994) Plant Physiol 105:89
102. Boller T, Gehri A, Mauch F, Vogeli U (1983) Planta 157:22
103. Linden JC, Phisalaphong M (2000) Plant Sci 158:41
104. Haigh JR (1988) MS thesis, Colorado State University
105. Sun X (1998) MS thesis Colorado State University
106. Mirjalili N (1997) PhD dissertation, Colorado State University

107. Phisalaphong M (1999) PhD dissertation, Colorado State University
108. Stanbury PF, Whitaker A (1984) Principles of fermentation technology. Pergamon Press, Oxford, p 95
109. Mizukami H, Ogawa T, Ohashi H, Ellis BE (1992) Plant Cell Rep 11:480
110. Xu Y, Chang PL, Liu D, Narasimhan ML, Raghothama KG, Hasegawa PM, Bressan RA (1994) Plant Cell 6:1077
111. Berry AW, Cowan DSC, Harpham NVJ, Hemsley RJ, Novikova GV, Smith AR, Hall MA (1996) Plant Growth Regul 18:135
112. Brailsford RW, Voesenek CJ, Blom CWP, Smith AR, Hall MA, Jackson MB (1993) Plant Cell Environ 16:1071
113. Schaller GE, Gamble RL, Randlett M, Zhao X, Qu X (2000) Ethylene receptors and the two-component paradigm. In: Walker J, Randall D (eds) Current topics in plant biochemistry, physiology and molecular biology. University of Missouri, Columbia, p 68
114. Rodriquez FI, Esch JJ, Hall AE, Binder BM, Schaller GE, Bleeker AB (1999) Science 283:996
115. Clark KL, Larsen PB, Chang C (1998) Proc Nat Acad Sci USA 95:5401
116. Chang C, Stewart RC (1998) Plant Physiol 117:723
117. Weber H, Vick BA, Farmer EE (1997) Proc Nat Acad Sci USA 94:10473
118. Fauth M, Schweizer P, Buchala A, Markst C, Riederer M, Kato T, Kauss H (1998) Plant Physiol 117:1380
119. Franke R, Fry SC, Kauss H (1998) Plant Cell Rep 17:379
120. Sessa G, Raz V, Savaldi S, Fluhr R (1996) Plant Cell 8:2223

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# Integrated Bioprocessing for Plant Cell Cultures

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Plant cell suspension culture has become the focus of much attention as a tool for the production of secondary metabolites including paclitaxel, a well-known anticancer agent. Recently, it has also been regarded as one of the host systems for the production of recombinant proteins. In order to produce phytochemicals using plant cell cultures, efficient processes must be developed with adequate bioreactor design. Most of the plant secondary metabolites are toxic to cells at the high concentrations required during culture. Therefore, if the product could be removed in situ during culture, productivity might be enhanced due to the alleviation of this toxicity. In situ removal or extractive bioconversion of such products can be performed by in situ extraction with various kinds of organic solvents. In situ adsorption using polymeric resins is another possibility. Using the fact that secondary metabolites are generally hydrophobic, various integrated bioprocessing techniques can be designed not only to lower toxicity, but also to enhance productivity. In this article, in situ extraction, in situ adsorption, utilization of cyclodextrins, and the application of aqueous two-phase systems in plant cell cultures are reviewed.

**Keywords.** Plant cell culture, In situ extraction, In situ adsorption, Cyclodextrin, Aqueous two-phase systems



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## 1 Introduction

The use of plant cells in a suspension culture as a tool for the production of a broad repertoire of secondary metabolites has been recognized for many years. The attempt to produce paclitaxel using plant cell cultures instead of extraction from intact plants is a good example of an industrial application [1]. Paclitaxel is now produced on a large scale by plant cell culture. Recently, plant cells have also been considered to be an alternative host for the production of recombinant proteins because they are able to glycosylate foreign proteins with biological activity [2]; it is easier to cultivate plant cells than animal cells on a large scale and they require simpler and cheaper media. However, the process is not competitive in comparison with intact plant production because of its low yield and unacceptable productivity. To be commercially viable, a selection of high-producing cell lines and efficient bioprocessing techniques should be combined.

Most of the secondary metabolites produced by plant cell cultures are hydrophobic and toxic to the cell growth. In addition, some of them are unstable. For this reason, the in situ removal of products has been proposed for the rapid recovery of volatile materials, thereby preventing the products from being de-

graded, avoiding end-product inhibition, or enhancing the secretion of such products [3]. Various methods have been designed to improve the recovery of secondary metabolites from plant cell cultures [4]. This in situ product removal enhanced the total secondary metabolite production and the products were selectively released from the cells. In situ adsorption using polymeric resins, in situ extraction with adequate solvents, application of cyclodextrins, and the utilization of aqueous two-phase systems are well-known examples of the results of combining cell culture and recovery processes. These combined processes make simultaneous cell growth and product recovery from a bioreactor feasible. Recently, the use of in situ recovery has been combined with other methods such as elicitation, immobilization, nutrient limitation, permeabilization, and growth promotion in order to increase the productivity further. Sometimes, synergistic effects were obtained [5]. These types of combined plant cell cultures are examples of what is referred to as integrated bioprocessing. Increasing the activity of metabolic pathways by elicitation, in conjunction with end-product removal or the accumulation of product in an extractive phase, has proven to be the most successful strategy [6]. The extractive phase added to the culture, whether it is solid or liquid, should be an inert hydrophobic chemical with a high adsorption capacity for the hydrophobic plant products. The adequate combination of strategies used may improve the production level by an order of magnitude. For the efficient enhancement of secondary metabolite production, the optimization of in situ recovery and the development of adequate processes are needed.

In this review, recent progress on integrated bioprocessing, including in situ adsorption using polymeric resins, in situ extraction with adequate solvents, application of cyclodextrins, and the use of aqueous two-phase systems, are summarized and their advantages and disadvantages are described in detail.

## 2
## In situ Extraction

### 2.1
### Integrated Bioprocessing with In situ Extraction

Secondary metabolites in plant cell culture are typically stored within the vacuolar compartments of the cells. Small amounts of metabolites are usually excreted into the medium or may appear in the medium due to cell lysis. In some cases, active transport of metabolites adjusts intracellular and extracellular levels in response to cellular conditions. Poor yields of secondary substances released into the medium may be caused by several factors. In those cases where low yield is due to cellularly mediated regulation of the ratio between intracellular and extracellular product concentrations, processes which reduce net medium concentrations such as enzymatic or non-enzymatic degradation, or volatility, should increase net production by depleting the level of the secondary substances in the culture medium. By using the so-called 'in situ extraction' method, the accumulation of a secondary substance inside the cell, in the culture medium, and in the extraction phase should approach an equilibrium, which

depend on the physicochemical properties of the material involved. It should be recognized, however, that an appropriate extraction phase for such accumulation of secondary metabolites must be tailored for each substance.

Both cell culture with a lipophilic extraction phase and with a polar extraction phase have been reported to be helpful for the accumulation and detection of secondary substances [7, 8]. Plant cell cultures release lipophilic and volatile substances such as ethylene, ethanol, and acetaldehyde. The addition of a lipophilic phase to the culture medium can be used as a means of accumulating and detecting these substances. Maisch et al. [8] found that the addition of XAD-4 resin to *Nicotiana tabacum* cultures enhanced the production of phenolic secondary metabolites several times compared to the adsorbent-free control. Kim and Chang [9] reported in situ extraction for enhanced shikonin production by *Lithospermum erythrorhizon*. When *n*-hexadecane was added to the cultivation, higher specific shikonin productivity was obtained than that from the cultures of free cells without extraction. They also suggested that *n*-hexadecane addition at an early stage in calcium alginate immobilized cell cultures was effective for shikonin production. Most of the produced shikonin was dissolved in *n*-hexadecane, so it would reduce the costs for shikonin separation.

### 2.2 In situ Extraction for the Enhanced Production of Secondary Metabolites in Plant Cell Cultures

Enhanced production of some of the benzophenanthridine alkaloids was found in an in situ extraction system that was composed of silicone fluid and a suspension culture of *Eschscholtzia californica* [10]. The time course changes of alkaloid production and distribution are shown in Fig. 1.

The alkaloid accumulation was expressed as a concentration based on the liquid volume of the indicated phase. In the extraction phase, it started to increase after 55 h and reached a maximum at 96 h. The concentration in the extraction phase at the maximum value was 25 times higher than that of the intracellular phase. The intracellular and extracellular concentration of alkaloid remained at values around those shown in Fig. 1. pH was an important factor for the in situ extractive culture of *E. californica*, as a pH elevation was observed in the media at the end of the culture and this corresponded to an increase in alkaloid accumulation in the extraction phase. In the in situ extraction system, alkaloid partitioning depends on the pH. The variation of the alkaloid partition coefficient in this system and over a given range pH is probably influenced by the ionic composition of the alkaloid molecule. Altering the pH changes the net charge of the alkaloid. The partition coefficient of sanguinarine, one of the benzophenanthridine alkaloids produced in *E. californica*, was found to be dependent on pH as shown in Fig. 2. This result demonstrates that pH elevation is one factor that can lead to an increase in alkaloid accumulation in the extracellular regions of the culture.

Elicitation in conjunction with an in situ extraction phase altered alkaloid production patterns. In particular, elicitors isolated from yeast extract can accelerate the production and the levels of benzophenanthridine alkaloids in *E.*

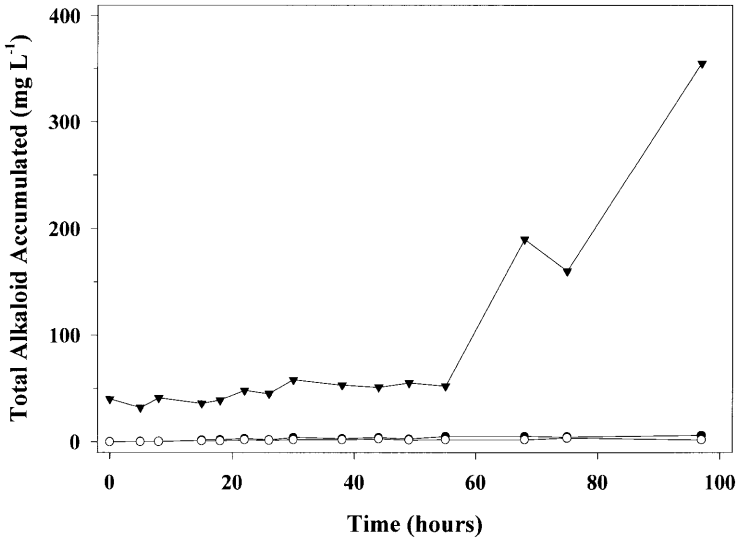



**Fig. 1.** Time course changes in the distribution of total alkaloid produced in in situ extractive cultures of *E. californica*. 23 vol.% of silicone fluid was used for in situ extraction. Symbols: (●) intracellular; (○) extracellular; (▼) in silicone fluid

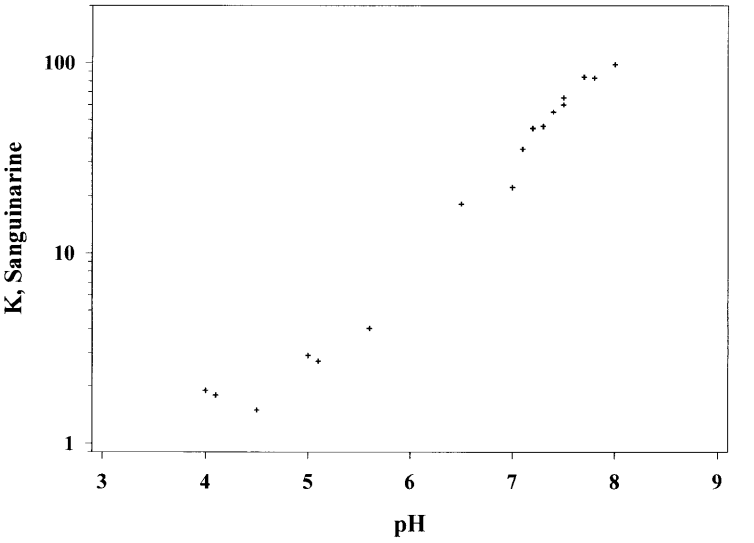



**Fig. 2.** pH effect on the partition coefficient (K) of sanguinarine, one of the benzophenanthridine alkaloids produced in cell cultures of *E. californica*, of in situ extraction with silicone fluid

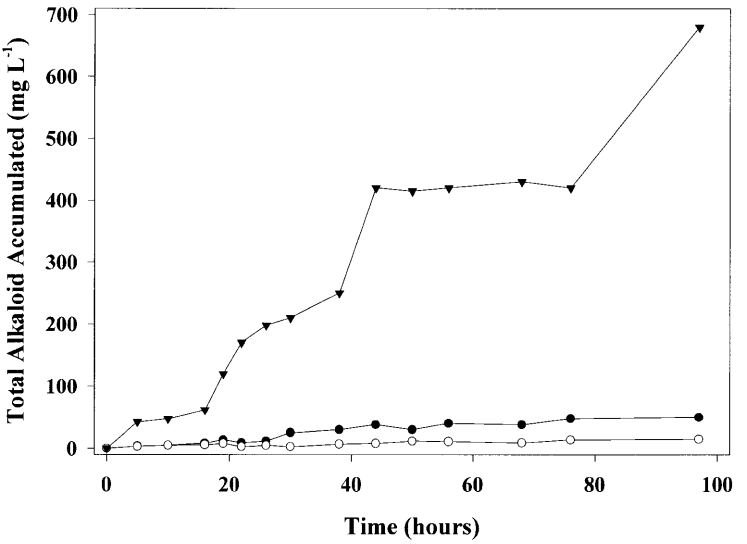



**Fig. 3.** Distribution of total alkaloid produced by in situ extraction with elicitation. 60 μg of yeast elicitor per gram of fresh cells was dosed at the same time 23% (v/v) of silicone fluid was added. Symbols: (●) intracellular; (○) extracellular; (▼) in silicone fluid

*californica* [11]. The time course behavior of elicitation in the in situ extraction culture of *E. californica* was examined. The alkaloid production and distribution are shown in Fig. 3. By eliciting the cultures, it was increased 2.5- to 10.5-fold. Elicitation also reduced the time required to reach high alkaloid concentrations. The combination of elicitation and in situ extraction culture presents several advantages over and above the individual methods. Elicitation in extractive culture clearly increased net alkaloid production as well as the concentration in the extraction phase.

### 2.3
### In situ Extraction in Airlift Bioreactor

In situ extraction for the enhanced production of secondary compounds can be applied in bioreactors. In bioreactor systems low productivity is an important bottleneck, and only a few products are potential candidates for economically feasible production of secondary metabolites using plant cell biotechnology. Several approaches to increase the productivity of secondary metabolites in bioreactors have been made [12]. Among them elicitation and in situ extraction are typical techniques of current interest. Application of these techniques to cell culture systems sometimes increased the productivity to such an extent that they have sometimes been viewed as the gateway to commercial success [10]. However, general rules or suggestions concerning these techniques cannot be made because of the different characteristics of the cell culture systems.

**Table 1.** Performance (total alkaloid production) comparison for each culture system

| Culture system (mg $g^{-1}$ dry cell) | Condition | Maximum Concentration (mg $L^{-1}$) | | | Volumetric productivity (mg $L^{-1}$ $day^{-1}$) | | | Net production (mg per fermentor) | | | Specific production Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Cellular | Silicone phase | phase | Cellular Total | Silicone phase | phase | Cellular Total | Silicone phase | phase | |
| Shake flasks | Control | 45.2 | – | 45.2 | 6.4 | – | 6.4 | – | – | – | 3.7 |
| Airlift fermentor 1 | Control | 14.0 | – | 14.0 | 0.7 | – | 0.7 | 32.2 | – | 32.2 | 2.1 |
| Airlift fermentor 2 | Elicitation | 16.1 | – | 16.1 | 0.8 | – | 0.8 | 37.0 | – | 37.0 | 2.7 |
| Airlift fermentor 3 | In situ extraction | 8.2 | 153.1 | 21.0 | 0.4 | 12.8 | 1.0 | 17.6 | 30.6 | 48.2 | 3.4 |
| Airlift fermentor 4 and elicitation | In situ extraction | 17.0 | 377.2 | 48.7 | 0.8 | 31.4 | 2.3 | 36.6 | 75.4 | 112.0 | 7.9 |

In a 2.3-l bench-top airlift bioreactor operation with *E. californica*, elicitation with an in situ extraction clearly increased net alkaloid production and concentration in the accumulation phase [13]. Typical advantages of elicitation with in situ extraction over two methods individually are as follows; it is believed to eliminate feedback inhibition by the secondary metabolites produced and to induce metabolic activities that increase secondary metabolite formation, whereas a single in situ extraction method does not. It is expected that the application of elicitation with in situ extraction can eliminate some major problems associated with poor yield of secondary metabolite production in plant cell culture. A summary of the performance of each culture system with *E. californica* is given in Table 1. It was found that the productivity for total alkaloid production in an airlift bioreactor was lower by one order of magnitude than that of normal shake flasks. Elicitation with in situ extraction improved total alkaloid production. The combination of elicitation and in situ extraction enhanced total alkaloid production synergistically. In an elicited in situ extractive airlift bioreactor operation the productivity and net production were 3.3 and 3.5 times higher than those of the normal airlift bioreactor operation. Although this increase was insufficient when compared to the large increases elicited in in situ extractive shake flask culture, elicitation with in situ extraction was a significant breakthrough for benzophenanthridine alkaloid production via airlift bioreactor operation, though productivity reductions were always associated with scale-up. Furthermore, it affords the in situ recovery of benzophenanthridine alkaloids in a specific manner that eliminates possible extractive separation processes with solvent. Optimization of operating conditions for this system can be expected to increase productivity further.

## 3
## In situ Adsorption

### 3.1
### In situ Product Recovery with Adsorbent

Although productivities may be improved by several culture techniques, plant cell product recovery may be complicated by the dilution of the target product in the medium. Depending on whether the target product is intracellular or extracellular, the harvested culture medium must be subjected to various separation and purification stages before the product of interest is isolated. The processes, which are commonly used, are cell removal, volume reduction, isolation, and purification until the purity of the product has achieved to the desired level. Therefore, in order to enhance process economics through improvements in the recovery stages, it is necessary to develop a separation technique which can concentrate the bioproduct, often with some degree of selectivity, and which is economical from the standpoints of capital requirements and operational cost in large scale cell culture. In addition to the above-mentioned issues, product separation can enhance the production of secondary metabolites by removing the feedback regulation mechanisms and nonspecific inhibitors in plant cell cultures [14, 15]. For these reasons, various attempts have been made

to achieve the in situ recovery of secondary metabolites from plant cell cultures [16, 17].

For the in situ product separation of plant cell culture, liquid-solid culture systems for plant cells consisting of an aqueous nutrient phase and polar solid adsorbents have been preferred because many products of plant cells are expected to be polar in character and bound weakly to the lipophilic phase of liquid-liquid systems. The differences in the acid-base properties and sorption characteristics of alkaloids further offer the potential for selectively absorbing specific alkaloids from a mixture.

Various adsorbents have been examined for their potential to increase in situ product separation in plant cell culture. Suspended solid adsorbents were popular, and the use of immobilized adsorbent has been investigated recently [17–20]. The advantages of immobilized adsorbent are that it is easy to use in a bioreactor operation and that it allows adsorbents to be easily separated from culture broth for the repeated use of cells and adsorbents [21, 22]. The design and optimization of in situ separation process for phytochemicals using immobilized adsorbent required a detailed mathematical model. It was difficult to achieve an optimal design based on purely empirical correlations, because the effects of various design parameters and process variables were coupled.

The use of solid adsorbents in combination with other processes has led to much progress in connection with increased metabolite production or bioconversion in plant cell culture. The integration of permeabilization and elicitation with in situ product separation by solid adsorbent stimulated de novo synthesis and bioconversion by the removal of products, preventing products being degraded or reducing feedback regulation [5, 23]. The integration of immobilized adsorbent and immobilized plant cells is also feasible in terms of continuous production in immobilized plant cell bioreactors involving the repeated use of cells [19, 20].

In this review the use of adsorbent for the in situ separation of product in plant cell culture is discussed, and a mathematical model which describes immobilized adsorbent coupled with selective separation is presented. Several examples of the application of the technique are also provided.

### 3.2
### Adsorbent

To increase metabolite production in plant cell culture, various adsorbents have been used for the solid/liquid two-phase system. Activated charcoal, lipophilic carrier (RP-8), zeolite, nonionic exchange resin (Amberlite XAD-2, XAD-4, XAD-7, XAD-8), acidic cationic exchange resins (Dowex 50 W, Amberlite IRC-50, IRC-200), basic anion exchange resin (Dowex i IX4–50, Amberlite IRA-93, IRA-400), mixed bed resin (TMD-8), and wofatite have all been examined as shown in Table 2. Before adsorbent is introduced to a vessel or bioreactor for this purpose, it should be pre-treated to activate the surface. As an example, the preparation of XAD-7 is detailed as follows.

**Table 2.** Examples of in situ adsorption with plant cell cultures

| Adsorbent | Cell cultures | References |
|---|---|---|
| Activated charcoal | *Matricaria chamomilla, Nicotiana tabacum, Vanilla fragrans* | 24–26 |
| RP-8 | *M. chamomilla, Mentha piperita, Pimella anisum, Valeriana wallichii* | 27, 28 |
| Zeolites | *N. tabacum* | 8 |
| Wofatite | *G. vernum* | 29 |
| XAD-2 | *Catharanthus roseus, Digitalis lanata, Galium vernum, Thuja occidentalis* | 23, 29, 30, 32, 33 |
| XAD-4 | *C. roseus, D. lanata, Nicotiana rustica, T. occidentalis, V. fragrans* | 23, 26, 31, 32, 33 |
| XAD-7 | *C. roseus, D. lanata, Thalictrum rugosum, V. fragrans,* | 15, 16, 18, 19, 20, 22, 23, 31, 32 |
| XAD-8 | *D. lanata* | 31 |
| Dowex 50 W, IX4–50 | *D. lanata* | 31 |
| IRC-50, IRC-200 | *D. lanata* | 31 |
| IRA-93, IRA-400 | *D. lanata* | 31 |
| TMD-8 | *D. lanata* | 31 |

### 3.2.1
### *Suspended Adsorbent*

Prior to use, 100 g of XAD-7 resin was soaked in methanol for 24 h and then washed with 2 l of distilled water. The washed resin was then air dried on filter paper under vacuum. Resins were sieved through nylon nets (mesh size: 500–900 μm) to obtain the required size distribution.

### 3.2.2
### *Immobilization of Adsorbent*

#### *3.2.2.1*
#### *Entrapped Adsorbent*

Two gram of alginic acid IV was dissolved in 98 g water by heating with stirring. 20 g XAD-7 was mixed with 80 g of the alginate solution at room temperature. Alginate beads of 4.35 mm diameter were made by dropping alginate/resin suspension into 1% $CaCl_2$ solution with continuous stirring. The beads were allowed to form for 30 min and then collected by filtration. They were washed with distilled water and autoclaved in 0.5% $CaCl_2$ solution for 15 min at 121 °C. The sterilized beads were stored in sterilized water.

#### *3.2.2.2*
#### *Encapsulated Adsorbent*

One gram of sodium alginic acid was dissolved in 99 ml water with heating and 20 g of XAD-7 was mixed with the alginate solution. The spherical calcium alginate gels were made by dropping the alginate/XAD-7 solution into 1.5% (w/v) calcium chloride solution. They were then allowed to form for 5 min and then collected by filtration. The beads were placed in 0.05% (w/v) poly-L-lysine solution for 5 min. A semi-permeable membrane of about 35 μm thickness was formed on the outer surface of the beads during this step. The capsules were then dropped into 1.5% (w/v) citric acid solution for 5 min to liquefy the core. The liquefied alginate diffused out into the bulk phase because the molecular weight of the alginate used (40,000–80,000 Dalton) is lower than the pore size of poly-L-lysine (100,000 Dalton). Capsules with 5.33-mm average diameter were formed and stored in distilled water.

### 3.2.3
### *Adsorption Characteristics*

Since the adsorption of plant metabolite onto adsorbent is a strong function of pH, the effect of pH upon the adsorption capacity of a plant metabolite should be investigated to understand and quantify product adsorption. The pH dependence of indole alkaloids, yohimbine and ajmalicine, and berberine onto XAD-7 has been reported [16, 20]. As a model system, XAD-7 was examined to quantify berberine adsorption, especially in terms of pH and concentration dependence, which were used for the in situ berberine separation in *Thalictrum rugosum* cultures [19, 20]. Dependence of berberine adsorption on pH and concentration was investigated as shown in Fig. 4. Equilibrium ratio (Q/C), an affinity of berberine, could be calculated by Eq. (1):

$$\frac{Q}{C} = \frac{(C_0 - C)}{C} \frac{V_B}{A_S} \tag{1}$$

where $C$ and $C_0$ were the measured and initial berberine concentrations, respectively, $V_B$ the liquid volume, $A_S$ the amount of adsorbent, and $Q$ was the adsorbent loading, that is the amount of berberine per unit mass of adsorbent (mg adsorbed/g adsorbent).

Figure 4 shows that the affinity increased with increasing pH up to 6 and then almost became constant. This result suggested that the uncharged form of berberine was adsorbed on XAD-7 because the aqueous concentration was the same as the concentration of the uncharged form over pH 6 due to the 2.47 $pK_a$ value of berberine.

If only neutral form of berberine could be adsorbed, the equilibrium on the adsorbent could be expressed as

$$C^+ \leftrightarrow C^0 + H^+ \tag{2}$$

$$C^0 + a_v \leftrightarrow C_a^0 \tag{3}$$

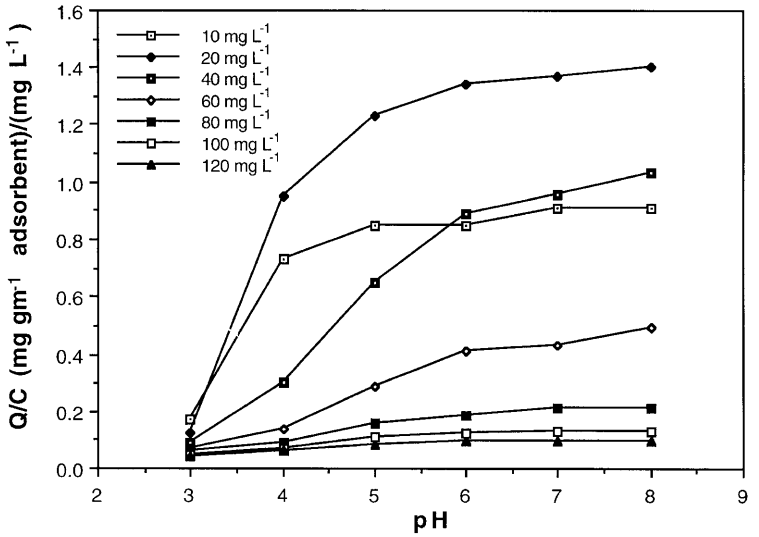



**Fig. 4.** Affinity of berberine adsorption on XAD-7 as a function of pH [20]

where $C^+$ and $C^0$ were the protonated and neutral forms of berberine, respectively, and $C_a^0$ and $a_v$ were the adsorbed berberine and sorption site, respectively. The total concentration of dissolved berberine, $C$, is

$$C = C^0 + C^+ \tag{4}$$

Based on the Hendersen-Hasselbalch relation [34], the protonated berberine concentration could be expressed as a function of the pH of the solution and the $pK_a$ of the berberine:

$$C^+ = C^0\, 10^{(pKa-pH)} \tag{5}$$

To find the neutral berberine concentration, Eq. (4) can be rearranged with Eq. (5):

$$C^0 = C/(1 + 10^{(pKa-pH)}) \tag{6}$$

Figure 5 shows the adsorbent loading, $Q$, as a function of $C^0$, based on data at various pHs. The result suggests that the neutral form of berberine was adsorbed on XAD-7. This result was consistent with the result that adsorption onto XAD-7 was due to hydrophobic interaction. In Fig. 5, the adsorption isotherm for berberine was of the Langmuir isotherm type. This result suggested that berberine might be adsorbed onto XAD-7 form a monolayer.

The selectivity of the target product to undesired product by encapsulated XAD-7 was examined by the competitive adsorption of berberine and dopamine produced in *Thalictrum rugosum* culture [18]. To investigate the degree of separation, the separation factor, $\alpha$, was considered:

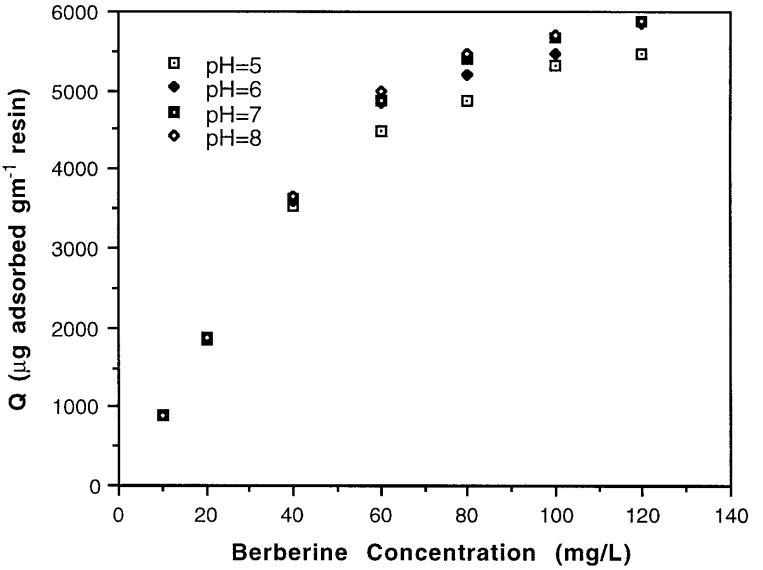



**Fig. 5.** Adsorption isotherm for berberine based on the concentration of the neutral form of berberine [7]

$$\alpha = \frac{\left(\frac{Q}{C_{bf}}\right)_{\text{Berberine}}}{\left(\frac{Q}{C_{bf}}\right)_{\text{Dopamine}}} \tag{7}$$

were

$$Q = \frac{(C_{b0} - C_{bf})V_B}{A_S} \tag{8}$$

where $C_{bf}$ is the final bulk concentration (mg $l^{-1}$), $A_s$ is the amount of capsules in the vessel ($g$), and $Q$ is capsule loading (mg adsorbed/g capsule).

The degree of separation was affected by pH as shown in Fig. 6. In the case of pure berberine solution, its affinity for XAD-7 increases with an increase in pH up to 6, and then becomes constant. This result suggests that the uncharged form of berberine was adsorbed on XAD-7 because the concentration of the neutral form of berberine was the same for pH values greater than 6, due to the 2.47 $pK_a$ value of berberine. For a pure dopamine solution, the affinity became constant with the increase in pH up to 7 and then decreased. Since dopamine has $pK_a$ values of 8.9 and 10.6, most of the dopamine is a negatively charged at pH values greater than 8.9. Only the uncharged form of dopamine is adsorbed on XAD-7 when the pH value is less than 8.9. As the pH of the mixed solution increases from 6 to 9, the separation factor increases because of the different $pK_a$ values of the berberine and dopamine.

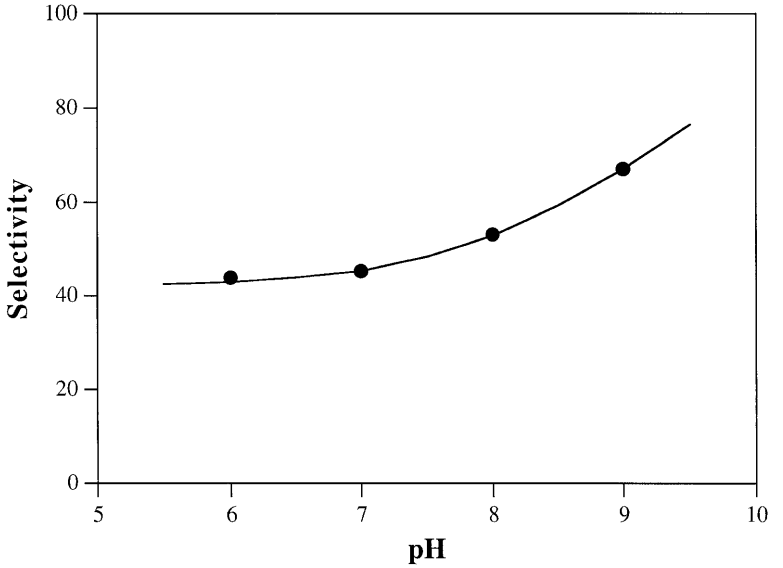



**Fig. 6.** Effect of pH on selectivity [18]

### 3.3
### In situ Separation with Adsorbent

#### 3.3.1
#### *In situ Separation with Suspended Adsorbent*

To remove the feedback regulation mechanism and to avoid product degradation various adsorbents have been used for the in situ separation of plant cell cultures as shown in Table 1. In situ removal with polymeric adsorbents stimulated anthraquinone production more than the adsorbent-free control in *Cinchona ledgeriana* cells [35]. It was found that nonionic polymeric resins such as Amberlite XAD-2 and XAD-4 without specific functional groups are suitable for the adsorption of plant metabolite [36]. The use of the natural polymeric resin XAD-4 for the recovery of indole alkaloids showed that this resin could concentrate the alkaloids ajmalicine by two orders of magnitude over solvent extraction [37] but the adsorption by this resin proved to be relatively nonspecific. A more specific selectivity would be beneficial because plant cells produce a large number of biosynthetically related products and the purification of a several chemically similar solutes mixture is difficult [16].

It was found that the polycarboxylic ester resin (XAD-7) was more selective in adsorbing indole alkaloids than XAD-4 despite a lower capacity [16]. When XAD-7 was added to the culture medium, the production of indole alkaloids was stimulated and increased in level compared to that of the undesired products. Selective recovery of ajmalicine, with the polycarboxyl ester resin XAD-7 has also been investigated. The accumulation of total indole alkaloids and the

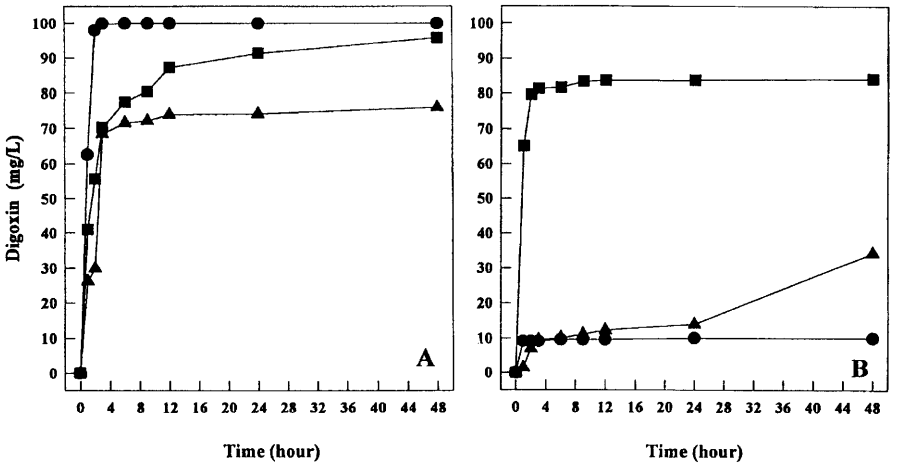



**Fig. 7 A, B.** Adsorption kinetics of digoxin on: **A** nonionic exchange resins (●, XAD-8; ■, XAD-4; ▲, XAD-7); **B** acidic exchange resins (●, IRC-200; ■, Dowex 50 W; ▲, IRC-50) [20]

specific alkaloids, ajmalicine and serpentine, was stimulated and an increased ratio of ajmalicine to serpetine was observed [15].

For the enhanced production of digoxin using in situ adsorption by bioconversion from digitoxin in plant cell suspension cultures, resin selection culture conditions were optimized as shown in Fig. 7 [31]. Among the various kinds of resins tested such as nonionic exchange resin (XAD-2, XAD-4, XAD-7, XAD-8) and acidic cationic exchange resins (Dowex 50 W, Amberlite IRC-50, IRC-200), XAD-8 was found to be the best for digoxin production in terms of adsorption characteristics and its the effect on cell growth. The optimum time for resin addition was determined to be 36 h from the initiation of the bioconversion and the contact time between the resin and the medium should be as short as possible to increase productivity.

To enhance alkaloid production and secretion in hair root cultures of *Catharanthus roseus*, a permeabilizing agent (dimethyl sulfoxide) and a fungal elicitor were treated together with in situ adsorption by XAD-7 [23]. By combining in situ adsorption with these techniques, the release ratio of catharanthine and ajmalicine was enhanced up to 20% and 70%, respectively, which was 3.4 and 2 times higher than that obtained with in situ adsorption by XAD-7 alone. Thus in situ adsorption sequentially applied with permeabilization and fungal elicitation showed a synergistic effect on the production and secretion of indole alkaloids.

The integration of elicitation, in situ product adsorption with XAD-7, and the immobilization of *Catharanthus roseus* cells lead to an increase in productivity and a significant increase in extracellular ajmalicine production [5]. The integration of in situ product separation by two-phase culture and immobilized plant cells could be feasible for continuous production in immobilized plant cell bioreactors requiring the repeated use of cells.

### 3.3.2
### *In situ Separation with Immobilized Adsorbent*

For the in situ product separation of berberine from immobilized *Thalictrum rugosum* cells, alginate-entrapped XAD-7 could be used to adsorb berberine [13, 20]. The advantages of immobilized adsorbent are that it is easy to use in a bioreactor operation and readily allows separation of adsorbents from the culture broth for the repeated use of cells and adsorbents [21, 22]. The permeabilization process was integrated with an in situ product separation process for intracellularly stored product in immobilized plant cell culture. Chitosan caused *Thalictrum rugosum* cells to secrete the intracellularly stored berberine by perturbing the cell membrane without damaging the cells [20]. Chitosan was used as permeabilizing agent and immobilized XAD-7 was used as a product adsorbent. In chitosan-treated cell culture to permeabilize intracellular berberine, berberine secretion was significantly accelerated by the addition of alginate-entrapped XAD-7 at the stationary phase of cell growth and thus more than 70% of the produced berberine was adsorbed by the alginate-entrapped XAD-7 as shown in Fig. 8.

The ability of encapsulated XAD-7 with a poly-L-lysine membrane was evaluated for the separation of product in the biotransformation by plant cells. The adsorption of digoxin by encapsulated XAD-7 was investigated in a biotransformation process from digitoxin into digoxin by *Digitalis lanata* cells. Encapsulated adsorbents were added to the culture medium on the second day of culture after the injection of digitoxin to separate the digoxin from the cell culture medium. After the addition of encapsulated adsorbent the digoxin concentration rapidly decreased due to the adsorption of digoxin onto the encapsulated adsorbent.

## 3.4
## Mathematical Model of Immobilized Adsorbent

The design and optimization of an in situ separation process for phytochemicals using immobilized adsorbent required a detailed mathematical model. It was difficult to achieve an optimal design based on purely empirical correlations, because the effects of various design parameters and process variables were coupled. A mathematical model based on reversible binding with local thermodynamic equilibrium assumptions for the competitive adsorption of phytochemicals on encapsulated adsorbents is proposed for the quantification of the adsorption phenomena and the evaluation of operating parameters, with respect to their effects on the selectivity of the target product. Design parameters for the optimal design of an in situ separation process for phytochemicals may be evaluated with the proposed model.

### 3.4.1
### *Model Equation*

The physical system and the associated notation are depicted in Fig. 9. The assumptions made to obtain the mathematical model were as follows: (i) adsorbent particles and capsules are spherical in shape; (ii) adsorbent particles

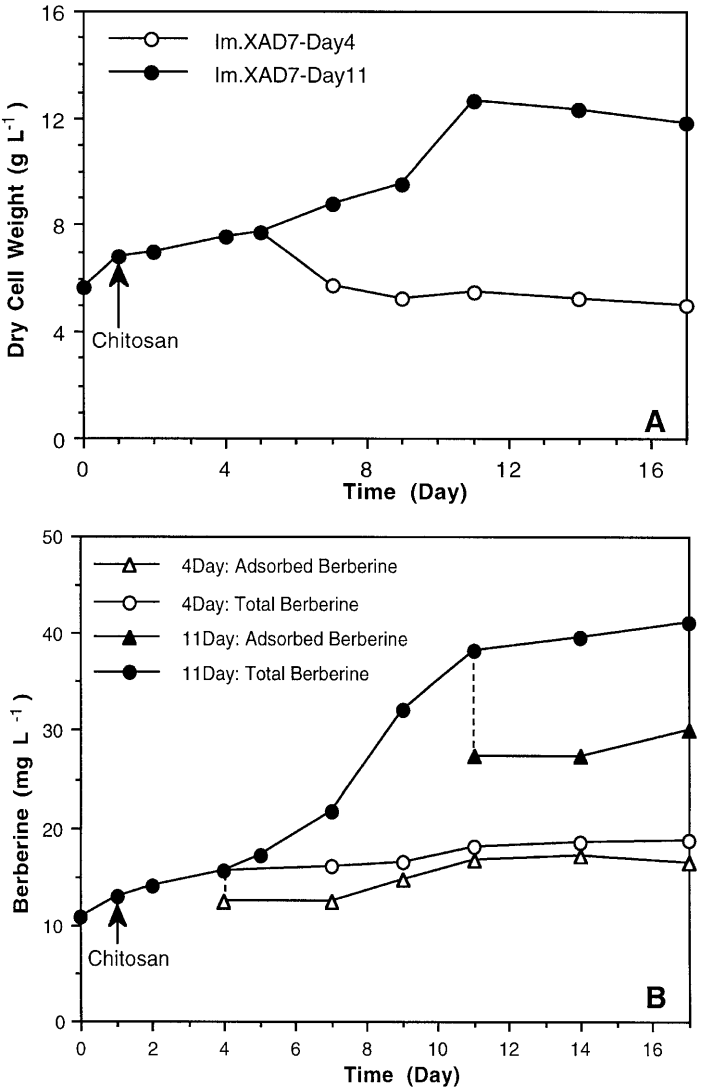



**Fig. 8 A, B.** Influence of immobilized XAD-7 on: **A** dry cell weight in chitosan-treated cell; **B** berberine production in chitosan-treated cell [20]

are distributed uniformly inside the capsule; (iii) the diffusion characteristics within the capsule, membrane and adsorbent particles are constant.

The governing Eqs. (9)–(12) were derived from balance equations obtained by considering the concentration in the core phase and the liquid (pore) phase of adsorbent [18]. Since the bulk phase is finite, the depletion in the bulk phase is

$$\frac{dC_{\mathrm{bi}}}{dt} = \frac{4\pi R_0^2 D_{\mathrm{mi}}}{V_{\mathrm{B}}} \left| \frac{\partial C_{\mathrm{mi}}}{\partial R} \right|_{\mathrm{R = R_0}} \tag{9}$$

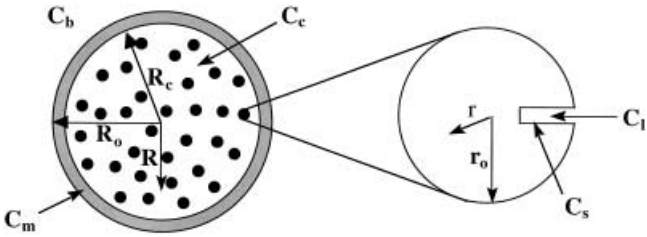



**Fig. 9.** Schematic diagram of an encapsulated adsorbent ($C_b$, concentration in bulk phase; $C_m$, concentration in membrane phase; $C_c$, concentration in capsule core; $C_l$, concentration in adsorbent pore; $C_s$, concentration in adsorbent surface; $R$, radial distance in capsule; $R_o$, radius of capsule membrane; $R_c$, radius of capsule core; $r$, radial distance in adsorbent; $r_o$, radius of adsorbent) [18]

where i is the index of adsorbing component, and $C_{bi}$ and $C_{mi}$ are the concentration of bulk and membrane phase, respectively. $R_0$ is the outer radius of capsule and $V_B$ is the bath volume, $D_{mi}$ is the diffusivity in the membrane and n is the adsorbent number.

Mass balance in the capsule gel membrane is given by

$$\frac{\partial C_{mi}}{\partial t} = \frac{D_{mi}}{R^2}\frac{\partial}{\partial R}\left[R^2\frac{\partial C_{mi}}{\partial R}\right] \tag{10}$$

Volume averaged homogeneous conservation equation for the capsule core phase is

$$\frac{\partial C_{ci}}{\partial t} = \frac{D_{ci}}{R^2}\frac{\partial}{\partial R}\left[R^2\frac{\partial C_{ci}}{\partial R}\right] - \frac{3N_s r_0^2 D_{li}}{R_0^3}\left|\frac{\partial C_{li}}{\partial r}\right|_{r=r_0} \tag{11}$$

where $D_{ci}$ and $D_{li}$ are the effective diffusivity in the capsule core and adsorbent pore, respectively, $N_s$ is the number of adsorbents per capsule, and $C_{ci}$ and $C_{li}$ are the concentrations in the adsorbent core phase and liquid (pore) phase, respectively.

The volume averaged homogeneous conservation equation in the adsorbent particle is

$$\frac{\partial C_{Ti}}{\partial t} = \frac{D_{li}}{r^2}\frac{\partial}{\partial r}\left[r^2\frac{\partial C_{li}}{\partial r}\right] \tag{12}$$

where $C_{Ti}$ is the total product concentration in the adsorbent, given by

$$C_{Ti} = C_{li} + m_{a_V} C_s \tag{13}$$

where $C_{li}$ and $C_{si}$ are the liquid (pore) and solid (surface) concentrations, respectively, and $a_V$ is the solid area available for adsorption. The void fraction is $\varepsilon_s$ and the parameter $m$ is

$$m = \frac{1-\varepsilon_S}{\varepsilon_S} \tag{14}$$

The initial concentrations are

$$C_{\mathrm{bi}} = C_{b0i}$$

$$C_{\mathrm{mi}} = C_{\mathrm{ci}} = C_{\mathrm{li}} = C_{\mathrm{si}} = 0 \quad \text{at } t = 0$$

The associated boundary conditions are

$$\left|\frac{\partial C_{\mathrm{ci}}}{\partial R}\right|_{\mathrm{R}=0} = \left|\frac{\partial C_{\mathrm{li}}}{\partial r}\right|_{\mathrm{r}=0} \tag{15}$$

$$D_{\mathrm{mi}}\left|\frac{\partial C_{\mathrm{mi}}}{\partial R}\right|_{\mathrm{R}=\mathrm{R}_{\mathrm{c}}^{+}} = D_{\mathrm{ci}}\left|\frac{\partial C_{\mathrm{ci}}}{\partial R}\right|_{\mathrm{R}=\mathrm{R}_{\mathrm{c}}^{+}} \tag{16}$$

$$C_{\mathrm{li}} = C_{\mathrm{ci}} \quad \text{at } r = r_0$$

$$C_{\mathrm{bi}} = C_{\mathrm{b0i}} \quad \text{at } R = R_0$$

These equations are supplemented by the equilibrium relationship between the solid and local liquid phase concentrations. Langmuir isotherm was chosen for the adsorption isotherm of a target product or by product:

$$C_{\mathrm{S1}} = \frac{C_{\mathrm{sm0}} \cdot C_{\mathrm{n}}}{K_{\mathrm{S0}} + C_{\mathrm{l1}}} \tag{17}$$

where $C_{\mathrm{sm0}}$ in the maximum solid phase concentration of a single component in adsorbent and $K_{\mathrm{S0}}$ is the adsorption equilibrium constant of a single component.

When two components are competitively adsorbed, the adsorption isotherms were rearranged as follows:

$$C_{\mathrm{S1}} = \frac{C_{\mathrm{l1}} C_{\mathrm{smT}}}{K_{\mathrm{S1}} + C_{\mathrm{l1}} + \dfrac{K_{\mathrm{S1}}}{K_{\mathrm{S2}}} C_{\mathrm{l2}}} \tag{18}$$

$$C_{\mathrm{S2}} = \frac{C_{\mathrm{l2}} C_{\mathrm{smT}}}{K_{\mathrm{S2}} + C_{\mathrm{l2}} + \dfrac{K_{\mathrm{S2}}}{K_{\mathrm{S2}}} C_{\mathrm{l2}}} \tag{19}$$

where $C_{\mathrm{smT}}$ is the maximum solid phase concentration for components, and $K_{\mathrm{S1}}$ and $K_{\mathrm{S2}}$ are equilibrium constants for target product and byproduct, respectively.

Furthermore, if external diffusion is important, the boundary condition between the bulk phase and membrane phase becomes

$$D_{\mathrm{mi}}\left|\frac{\partial C_{\mathrm{mi}}}{\partial R}\right|_{\mathrm{R}=\mathrm{R}_0} = -\,k_{\mathrm{Li}}\,(C_{\mathrm{mi}} - C_{\mathrm{bi}}) \tag{20}$$

where $k_{\mathrm{Li}}$ is the mass transfer coefficient.

In dimensionless form, Eqs. (9) – (12) are reduced to

$$\frac{d\phi_{\mathrm{bi}}}{d\theta_{\mathrm{i}}} = -\,3\,\frac{\beta}{\gamma_{\mathrm{i}}} N_{\mathrm{R}}\left|\frac{\partial \phi_{\mathrm{mi}}}{\partial R}\right|_{\bar{\mathrm{R}}=1} \tag{21}$$

$$\frac{d\phi_{\mathrm{mi}}}{d\theta_{\mathrm{i}}} = \frac{\beta}{\gamma_{\mathrm{i}}} \frac{1}{\bar{R}^2} \frac{\partial}{\partial \bar{R}} \left[ \bar{R}^2 \frac{\partial \phi_{\mathrm{mi}}}{\partial R} \right] \tag{22}$$

$$\frac{d\phi_{\mathrm{ci}}}{d\theta_{\mathrm{i}}} = \beta \left[ \frac{1}{\bar{R}^2} \frac{\partial}{\partial \bar{R}} \left[ \bar{R}^2 \frac{\partial \phi_{\mathrm{ci}}}{\partial R} \right] - 3\, N_{\mathrm{s}} \delta_{\mathrm{i}} \sqrt{\beta} \left( \frac{\partial \phi_{\mathrm{li}}}{\partial r} \right)_{\bar{\mathrm{r}}=1} \right] \tag{23}$$

$$\frac{d\phi_{\mathrm{li}}}{d\theta_{\mathrm{i}}} = \frac{\delta}{\Gamma(\phi_{\mathrm{li}})} \frac{1}{\bar{r}^2} \frac{\partial}{\partial \bar{r}} \left[ \bar{r}^2 \frac{\partial \phi_{\mathrm{li}}}{\partial r} \right] \tag{24}$$

where, for competitive adsorption $\Gamma(\phi_{\mathrm{li}})$ is

$$\Gamma(\phi_{\mathrm{li}}) = 1 + \frac{m\phi_{\mathrm{sm1}}\omega_1}{\left( \omega_1 + \phi_{\mathrm{l1}} + \dfrac{\phi_{\mathrm{l2}}\omega_1}{\omega_2} \right)^2} \tag{25}$$

$$\Gamma(\phi_{\mathrm{l2}}) = 1 + \frac{m\phi_{\mathrm{sm1}}\omega_2}{\left( \omega_2 + \phi_{\mathrm{l2}} + \dfrac{\phi_{\mathrm{l1}}\omega_2}{\omega_1} \right)^2} \tag{26}$$

The initial concentrations are

$$\phi_{bi} = 1 \qquad \text{for } \theta_i = 1$$

$$\phi_{mi} = \phi_{ci} = \phi_{li} = \phi_{si} = 1 \qquad \text{for } \theta_i = 1$$

The boundary conditions are

$$\left| \frac{\partial \phi_{\mathrm{ci}}}{\partial R} \right|_{\bar{\mathrm{R}}=0} = \left| \frac{\partial \phi_{\mathrm{li}}}{\partial r} \right|_{\bar{\mathrm{r}}=0} = 0 \tag{27}$$

$$\left| \frac{\partial \phi_{\mathrm{mi}}}{\partial \bar{R}} \right|_{\bar{\mathrm{R}}=\bar{\mathrm{R}}_{\mathrm{c}}^{+}} = \gamma_{\mathrm{i}} \left| \frac{\partial \phi_{\mathrm{ci}}}{\partial \bar{R}} \right|_{\bar{\mathrm{R}}=\bar{\mathrm{R}}_{\mathrm{c}}} \tag{28}$$

$$\left| \phi_{\mathrm{ci}} \right|_{\bar{\mathrm{r}}=1} = \phi_{\mathrm{ci}}$$

$$\left| \phi_{\mathrm{bi}} \right|_{\bar{\mathrm{R}}=1} = 1$$

where $V_{\mathrm{H}}$ is the total volume of capsule.

The dimensionless variable $\phi_{\mathrm{i}}$ is introduced by normalizing the corresponding concentration $C_{\mathrm{i}}$ to the initial product concentration $C_{0\mathrm{i}}$. The other dimensionless groups are defined as

$$\theta_{\mathrm{i}} = \frac{C_{\mathrm{ci}} t}{r_0^2};\ \bar{R} = \frac{R}{R_0};\ \bar{R}_{\mathrm{C}} = \frac{R_{\mathrm{C}}}{R_0};\ \bar{r} = \frac{r}{r_0};$$

$$\beta = \frac{r_0^2}{R_0^2};\ \gamma_{\mathrm{i}} = \frac{D_{\mathrm{ci}}}{D_{\mathrm{mi}}};\ N_{\mathrm{R}} = \frac{nV_{\mathrm{H}}}{V_{\mathrm{B}}};\ \delta_{\mathrm{i}} = \frac{D_{\mathrm{li}}}{D_{\mathrm{ci}}}$$

The dimensionless parameters that define the particular problems are

$$\omega_i = \frac{K_{Si}}{C_{\phi i}}\ ;\ m\phi_{smi} = \frac{C_{smT}\alpha_v}{C_{\phi i}}$$

The parameters, $C_{smT}$, $K_{S1}$, and $K_{S2}$ are estimated for competitive adsorption. The model is reduced to the functional form $\phi_i = \phi(\theta_i; m\phi_{smi}, \omega_i)$.

### 3.4.2 *Numerical Techniques*

For encapsulated adsorbent, it is convenient to introduce the transformation $\xi = \bar{R}^2$ and $\xi = \bar{r}^2$ to eliminate Eq. (27), and the two point nature of the boundary conditions.

For the product concentration in the adsorbent, let

$$\frac{\partial \phi_{li,f}}{\partial \xi} = \sum_{g=1}^{L+1} A1_{fg}\phi_{li,g}\ ;\quad \frac{\partial^2 \phi_{li,f}}{\partial \varsigma^2} = \sum_{g=1}^{L+1} B1_{fg}\phi_{li,g}$$

be approximations to the corresponding derivatives at the location $\zeta_i$ that are the roots of an L-th-order Jacobi polynomial.

For the product concentration in the capsule core, let

$$\frac{\partial \phi_{ci,p}}{\partial \xi} = \sum_{p=1}^{M+1} A2_{pg}\phi_{ci,p}\ ;\quad \frac{\partial^2 \phi_{ci,p}}{\partial \xi^2} = \sum_{q=1}^{M+1} B2_{pg}\phi_{ci,p}$$

be approximations to the corresponding derivatives at the location $\xi_i$ that are the roots of an M-th-order Jacobi polynomial.

For the product concentration in the hydrogel membrane, let

$$\frac{\partial \phi_{mi,u}}{\partial \xi} = \sum_{V=0}^{N+1} A3_{uv}\phi_{mi,v}\ ;\quad \frac{\partial^2 \phi_{mi,u}}{\partial \xi^2} = \sum_{v=0}^{N+1} B3_{uv}\phi_{mi,v}$$

be approximations to the corresponding derivatives at the location $\xi_i$ that are the roots of an *N*-th-order Jacobi polynomial.

The evaluation of these elements and the underlying theoretical support for the method can be found in Villadsen and Michelsen [38] who also provided subroutine listings that were used in this study. The boundary condition for the adsorbent particles is $\phi_{li,L+1} = \phi_{ci}$ where *L* is the number of internal collocation points that corresponds to a particular *L*-th-order polynomial approximation. The boundary condition for the capsule core is $\phi_{ci,M+1} = \phi_{mi,0}$ where *M* is the number of internal collocation points that correspond to a particular *M*-th-order polynomial approximation, and the boundary condition for the hydrogel membrane is $\phi_{mi,N+1} = \phi_{bi}$ where *N* is the number of internal collocation points that corresponds to a particular *N*-th-order polynomial approximation. Since the boundary conditions for the adsorbent and capsule core are coupled, and that of the capsule core and hydrogel membrane are also coupled, the boundary

condition can be expressed as $\phi_{bi} = \phi_{mi,N+1}$, $\phi_{mi,0} = (\phi_{ci,M+1})_0 = ((\phi_{li,L+1})_{M+1})_0$. Substituting the above relationships into the diffusion equations and Eq. (28) yields a set of first-order differential equations as follows:

$$\frac{d\phi_{li,f}}{d\theta_i} = \frac{\delta_i}{\Gamma(\phi_{li})} = \sum_{g=1}^{L+1} E_{fg}\phi_{li,g}, \quad f = 1 \sim L \tag{29}$$

where

$$E_{fg} = 6A1_{fg} + 4\zeta_f B1_{fg} \tag{30}$$

For the capsule core,

$$\frac{d\phi_{ci,p}}{d\theta_i} = \frac{d[\phi_{li,L+1}]_P}{d\theta_i} = \beta\left(\sum_{q=1}^{M+1} F_{pg}\phi_{ci,q} - 6\,N_S\delta_i\sqrt{\beta}\sum_{g=1}^{L+1} F_{pg}\phi_{ci,q}\right) \tag{31}$$
$$f = 1 \sim L,\ P = 1 \sim M$$

where

$$F_{pq} = 6A2_{pq} + 4\xi_p B2_{pq} \tag{32}$$

For the capsule membrane,

$$\frac{d\phi_{mi,u}}{d\theta_i} = \frac{\beta}{\gamma_i}\sum_{v=1}^{N+1} G_{uv}\phi_{mi,v}, \quad u = 1 \sim N \tag{33}$$

where

$$G_{uv} = 6A3_{uv} + 4\xi_f B3_{uv} \tag{34}$$

For bulk phase

$$\frac{d\phi_{bi}}{d\theta_i} = \frac{d[\phi_{mi,N+1}]}{d\theta_i} = -\frac{6\beta}{\gamma_i} N_P \sum_{v=1}^{N+1} A3_{uv}\phi_{mi,v} \quad u = 0 \sim N \tag{35}$$

And for boundary condition at the interface between membrane and core,

$$\sum_{v=0}^{N+1} A3_{0v}\phi_{mi,v}; \quad \gamma_i \sum_{q=1}^{M+1} A2_{M+1,q}\phi_{ci,p} \tag{36}$$

that are easily integrated by an explicit Runge-Kutta method [38] with the initial conditions $\phi_{li,f}(0) = 0$, $\phi_{ci,p}(0) = 0$, $\phi_{mi,u}(0) = 0$, $\phi_{bi}(0) = 1$.

When the relative volumes are known and the diffusion coefficients in the capsule core and capsule membrane can be estimated a priori in single component adsorption, the parameter to work with is the effective diffusivity in the adsorbent pore ($D_{11}$). Then, with the above estimated parameter values, the parameters of competitive adsorption are the maximum concentration at the solid phase of the adsorbent ($C_{smT}$), and the equilibrium constants of the target product ($K_{S1}$) and byproduct ($K_{S2}$).

The solution to Eqs. (29), (31), (33), (35), and (36) depends on the choice of $L$, $M$, $N$, and the Jacobi polynomial used for the basic function. The Jacobi polynomial used in all numerical approximations is characterized by the weighting factor $\zeta^{1/2}(1-\zeta)$ over the interval $0<\zeta<1$ and $\xi^{1/2}(1-\xi)$ over the interval

$0 < \xi < 1$. $L = 4$ for the adsorbent particle, $M = 4$ for the capsule core, and $N = 2$ for the capsule membrane were chosen as the number of internal collection points, which represents a compromise between extreme accuracy and computational speed. The calculation steps are as follows. When the collection point in the capsule is being calculated, the value of each point is determined using the values of the adsorbent surface which, in turn, were calculated from the whole collection point in the adsorbent. $\phi_{li}$ was calculated first, then $\phi_{ci}$ was calculated based on $\phi_{li,L+1} = \phi_{ci}$. $\phi_{mi}$ was then calculated based on $\phi_{mi,0} = (\phi_{ci,M+1})_0 = ((\phi_{li,L+1})_{M+1})_0$ and $\phi_{bi}$ was calculated based on $\phi_{bi} = \phi_{mi,N+1}$.

The experimental data, $C_{bi}$ vs time, were compared to the model predictions by choosing the parameters $m\phi_{smi}$ and $\omega_i$ that gave the best fit with the competitive adsorption data. A nonlinear parameter estimation package was used [39] which employed a weighting factor for the residuals proportional to $\phi^{-2}$ so that the information obtained near equilibrium conditions was highlighted.

### 3.4.3 *Model Validation*

As trial system to test the application of the proposed model the ability of encapsulated XAD-7 was evaluated for the selective separation of berberine from dilute aqueous mixtures of berberine and dopamine, the target secondary metabolite, and an undesirable intermediate metabolite of *Thalictrum rugosum* plant cell culture [18]. Competitive adsorption experiments were performed in dilute aqueous mixtures of berberine and dopamine, both at initial concentrations of 60 mg $l^{-1}$, which is representative of actual plant cell culture. Experimental and theoretical results for normalized bulk concentration profiles of berberine and dopamine are shown in Fig. 10. The bulk berberine concentration was reduced to approximately 4.6% of the initial concentration, which indicates that 95.4% of the berberine in the initial mixed solution was adsorbed. Encapsulated XAD-7, therefore, selectively concentrated the berberine from dilute aqueous mixtures of berberine and dopamine.

### 3.4.4 *Simulation Study for the Effects of Design Parameters*

The proposed mathematical model for encapsulated adsorbents can describe various diffusion characteristics in addition to the intrinsic binding characteristics of the encapsulated adsorbents. The performance of encapsulated adsorbent in an in situ product separation process can be evaluated using the proposed model for the adsorption rate of a target product, berberine. The performance of the encapsulated adsorbents is influenced by design parameters such as the adsorbent content in the capsule ($N_s$), the capsule size ($R_0$), the number of capsules ($n$), the membrane thickness ($R_m$), and the ratio of the single capsule volume to the total capsule volume ($N_c$).

Figure 11 shows the change in the bulk concentrations of berberine and dopamine when the design parameters were changed twofold compared to the control value. Adsorption curves for $N_s$, $R_0$, $n$, $R_m$, and $N_c$ are compared to that

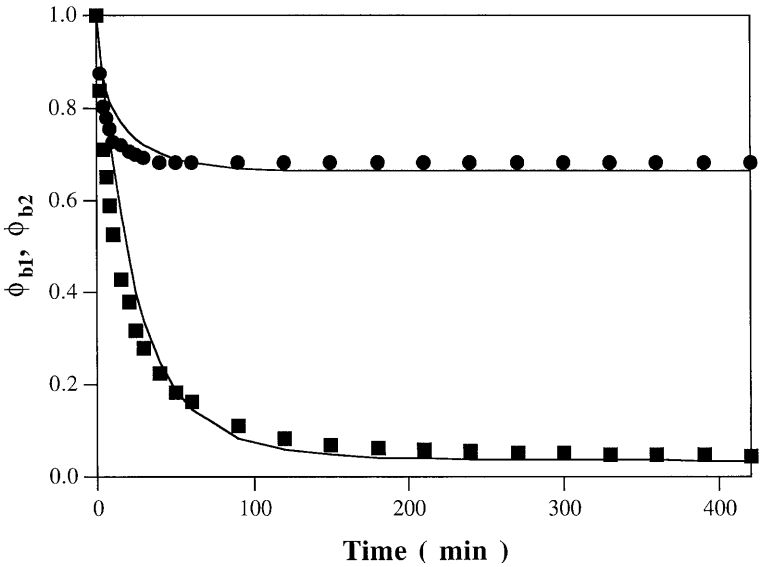



**Fig. 10.** Experimental (*symbol*) and theoretical (*line*) results for the competitive adsorption of berberine and dopamine on encapsulated adsorbent (●, 60 mg $l^{-1}$ berberine and ■, 60 mg $l^{-1}$ dopamine) [18]

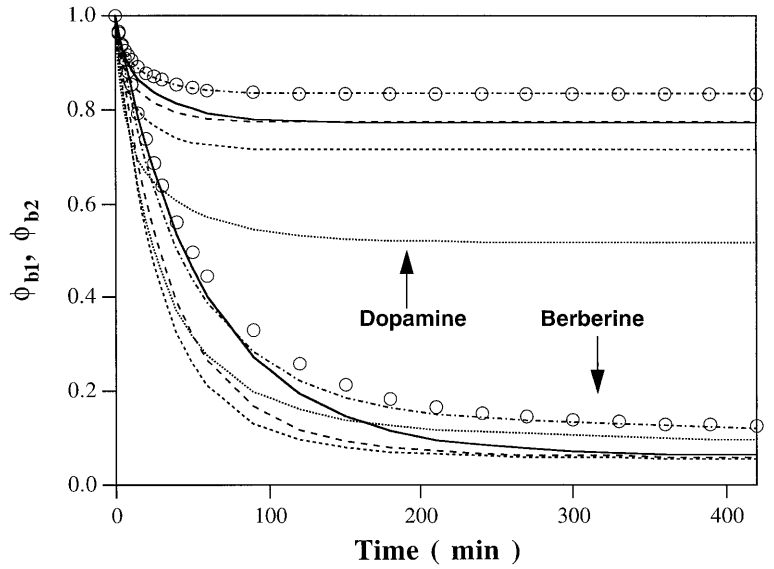



**Fig. 11.** Simulated bulk concentration profiles for the effect of design parameters on the adsorption of berberine and dopamine on encapsulated adsorbent (○, control condition; ---, change of $N_s$; ···, change of $R_o$, ---, change of $n$; -·-·-·-, change of $R_m$; —, change of $N_c$) [18]

**Table 3.** Effect of design parameters on selectivity [18]

| Design parameters | Separation factor |
|---|---|
| Control | 34.949 |
| Adsorbent content | 50.047 |
| Capsule size | 10.324 |
| Capsule number | 43.832 |
| Membrane thickness | 36.260 |
| Ratio of single capsule volume to total capsule volume | 55.785 |

of control. The adsorption kinetics is enhanced compared to the control case for every parameter change in terms of rate and loading capacity. The highest increase of adsorption rate and capacity for berberine alone are observed by increasing the capsule number, but in this case the separation factor is low due to the significant increase in dopamine adsorbed. Table 3 presents a comparison of the separation factor for the various design parameters. Based on the calculated separation factor, the ratio of single capsule volume to the total capsule volume was the most effective design parameter which could be used to separate berberine selectively.

Thus, the proposed mathematical model for the encapsulated adsorbent can be used to help produce an optimal design for the separation of phytochemicals by evaluating design parameters. However, since the optimization of design parameters depends on specific process characteristics, it is relatively difficult to obtain the optimal design parameters based purely on empirical correlations. The proposed mathematical model can evaluate the design parameters in various process conditions. When there are one or more compounds present in the fermentation broth, which compete for the adsorption site in the adsorbent particle, the proposed model can be used to evaluate the design parameters and maximize the selectivity of the desired product by introducing adsorption rate constants for the various products.

### 3.4.5 *Combined Model with Kinetics and Adsorption Model*

In situ separation of phytochemicals by plant cell culture was analyzed using a combination of the proposed model for encapsulated adsorbent using the local thermodynamic equilibrium isotherm and the kinetic model of cell growth and product formation. A biotransformation process for the production of digoxin by *Digitalis lanata* cell culture was chosen as a model system. Digoxin is one of the cardiac glycosides (cardenolides), which are important pharmaceuticals in the treatment of heart diseases. The biotransformation from digitoxin into digoxin in the *Digitalis lanata* cell culture has been investigated, as has the kinetic model of cell growth and product formation [40, 41]. The combined model for in situ digoxin separation with kinetic model and adsorption model is solved simultaneously. Figure 12 shows the experimental and model predicted cell growth. The proposed model predicts experimental results fairly well. The mathematical model combined with the adsorption model and the kinetic model

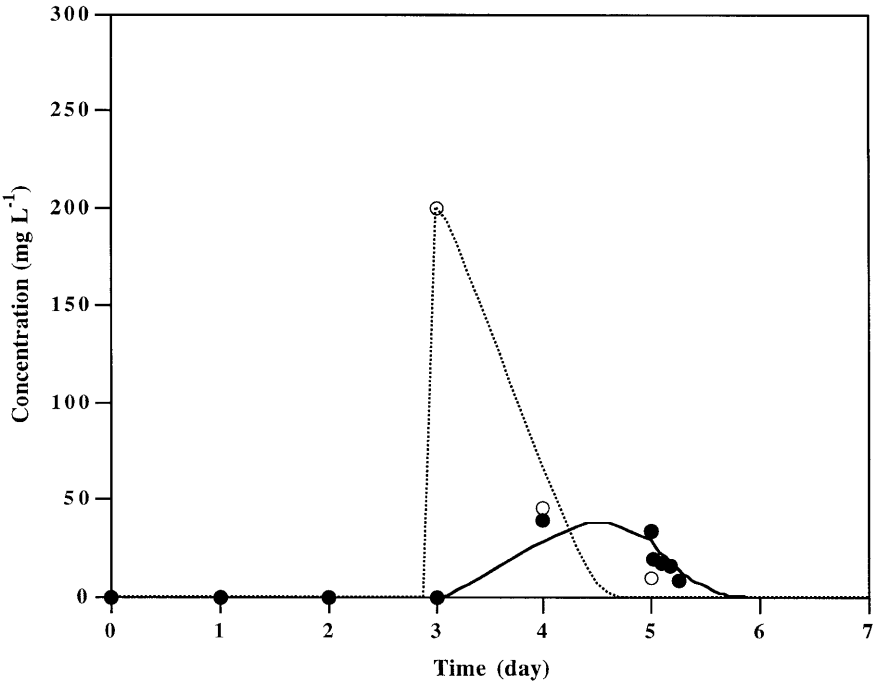



**Fig. 12.** Experimental (*symbol*) and model predicted (*line*) results of in situ separation of digoxin from the *Digitalis lanata* K3OHD cell culture medium (●, digoxin; ○, digitoxin) [unpublished results]

can be applied to analyze the performance and to design a system for in situ product separation in plant cell culture.

## 4
## Cyclodextrins in Plant Cell Cultures as Product Enhancers

### 4.1
### Cyclodextrins

Recently, cyclodextrins have received considerable attention because of their ability to form stable inclusion complexes with organic molecules [42–44]. The schematic configuration of complex formation with a guest molecule is shown in Fig. 13. Their ability to form stable complexes with a wide spectrum of guest molecules principally leads to enhancement of the solubility of guest molecules in the aqueous environment, which enables cyclodextrins to be used in various applications. The natural cyclodextrins are cyclic oligosaccharides consisting of 6, 7, or 8 D-glucopyranosyl units connected by $\alpha$-1,4 glycosidic linkages and produced commonly by the biotransformation of starch by the bacterial enzyme cyclodextrin glycosyltranferase. The most interesting structural feature of cyclodextrin molecules is that they possess a molecular cavity, the interior of

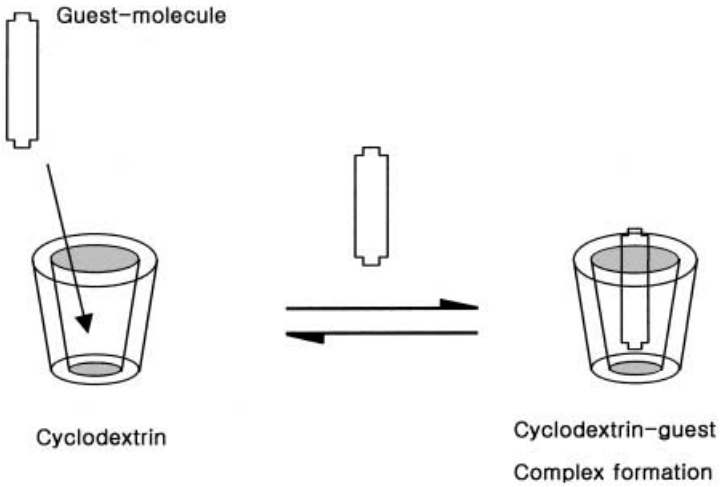



**Fig. 13.** Schematic representation of complex formation of cyclodextrin with guest molecule

which is hydrophobic as a result of the electron rich environment provided to a large extent by the glycosidic oxygen atoms.

The characteristics of these interesting molecules have been exploited in various areas of applications such as pharmaceuticals, food and the separation of enantiomers, etc. First of all, we would like to review some examples of applications other than those involving plant cells, which demonstrate the basic principles and ideas associated with improved productivity in plant cell applications.

Pharmaceutical applications of cyclodextrins have been studied the most extensively. The identified roles of cyclodextrins are primarily to increase solubility, stability, and the bioavailability of drugs with limited water solubility, to reduce the side effects and toxicity of drugs, and to alter their physical properties such as smell and taste. There have been many excellent reviews on the pharmaceutical utilization of cyclodextrin and its derivatives by Loftsson and Brewster [45], Rajewski and Stella [46], and Irie et al. [47].

Application of cyclodextrins can be also found in cell cultures. Cyclodextrin was used to remove unwanted cholesterol from cultures of L-cell mouse fibroblasts [48].

Similarly, removal of cholesterol from liquid egg yolk using $\beta$-cyclodextrin has been reported. This involved the formation of an insoluble complex by binding with cholesterol in the oil-water interface of egg yolk. As the amount of added $\beta$-cyclodextrin increased, the cholesterol removal efficiency was increased but valuable nutrients of egg yolk also decreased. As a result, it was concluded that the molar ratio of 3:1~5:1 for $\beta$-cyclodextrin:cholesterol was most effective at removing cholesterol from egg yolk [49].

Cyclodextrins were investigated as media supplements in liquid cultures of *Helicobacter pylori*, in which the culture requires the addition of other media supplements that often interfere with the subsequent purification of bacterial

antigens. Several commercial cyclodextrins added to Brucella broth supported a flourishing growth and permitted the consistent production of vacuolating cytotoxin [50].

Cyclodextrins and modified cyclodextrins are widely used as chiral selectors [51]. For an example, the enantiomers of atropine, a main ingredient of *Scopolia* extract and *Scopolia rhizome*, was separated by capillary electrophoresis with cyclodextrin media [52].

The book written by Froamming and Szejtli [53] provides an overview on cyclodextrin and its application.

### 4.2 Applications in Plant Cell Cultures

Many plant secondary metabolites produced by cell cultures and substrates used in culture media are often hydrophobic and have low solubility in aqueous medium. Improving the production of metabolites in the plant cell system has also been attempted by enhancing the solubilities of metabolites by using the cyclodextrin-guest complex formation. There are two major approaches, one involves the improvement of substrate solubility and the other improves metabolite.

Addition of $\beta$-cyclodextrin to plant cells was quite successful in biotransformation which required precursors with poor solubility in culture media. An attempt was made by Ramachadra and Ravishanker [54] to study the biotransformation of isoeugenol to vanilla flavor metabolites and capsaicin under the influence of $\beta$-cyclodextrin and fungal elicitor in immobilized cell cultures of *C. frutescens.* Isoeugenol is an isomerization product of eugenol, and was used as the starting material in the manufacture of vanillin [55]. Biotransformation of isoeugenol in the immobilized cells was enhanced by the addition of $\beta$-cyclodextrin, which did not affect cell growth, but vanillin production nevertheless showed a 1.62-fold increase over cultures treated with isoeugenol alone. They speculated that the increased accumulation of vanillin was possibly due to the solubility enhancement of the precursor, isoeugenol. A similar result was reported in hydroxylation of 17-estradiol to 4-hydroxy estradiol in *Mucuna pruriens* cell cultures, in which the transformation reached 40% in the presence of $\beta$-cyclodextrin while no bioconversion was observed without $\beta$-cyclodextrin [56]. In suspension cultures of *Digitalis lanata*, the addition of $\beta$-cyclodextrin increased the solubility and stability of digitoxin, which is a precursor of digoxin, and the digoxin production was substantially enhanced [57]. Similarly, the production of digoxin was enhanced 4.5-fold by the addition of $\beta$-cyclodextrin to suspension cultures of *C. frutescens* compared with digitoxin alone [58].

A number of articles have been written which concern the poor solubilities of products. Suspension cultures of *Mentha* produce menthol which has very low solubility in water due to its hydrophobicity, and this is considered to be a factor responsible for its low production in the suspension cultures. Cyclodextrin has a hydrophobic cavity inside the molecule in which menthol can be captured and allow to form a stable complex. A suspension culture of *Mentha piperita* showed a 70% production enhancement in a medium contain-

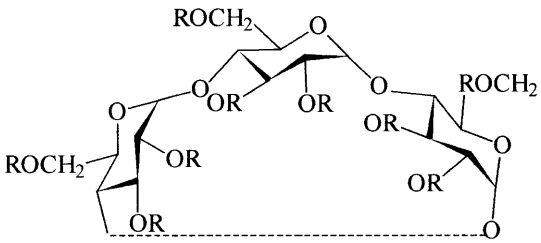
ing 1.5%(w/v) $\beta$-cyclodextrin compared to the cultures without cyclodextrin [59]. $\beta$-Cyclodextrin had no adverse effect on the cell growth and showed the best result amongst the $\alpha$-, $\beta$-, and $\gamma$-cyclodextrins tested in terms of menthol production. Similarly, the addition of $\beta$-cyclodextrin to culture media also enhanced the amount of monoterpenes accumulated in shoot cultures of *Mentha canadensis* and menthol; in particular, they accumulated up to 50% more total essential oil [60].

Park and Cho [61] reported an extractive production system for benzophenanthridine alkaloids using cyclodextrins, in which production and some degree of separation occurred simultaneously. It was expected that the rapid removal of alkaloid produced from the suspension cultures was achieved by capturing alkaloid with cyclodextrins. In solid cultures of *E. californica* cells, alkaloid production was substantially enhanced (up to 40-fold) by the addition of $\beta$-cyclodextrin. The enhancement of alkaloid production was also observed in suspension cultures, in which the major part of the alkaloids in the $\beta$-cyclodextrin treated cultures was present in the media, while the non-treated cultures contained the alkaloids in the cells. This indicated that $\beta$-cyclodextrin not only captures the alkaloids but also helps in extracting them from cells.

### 4.3 Modification of Cyclodextrin

Modification of the parent cyclodextrins might be required to improve their ability to form inclusion complexes with various substrates, and enhance their selectivity to guest molecules. An attempt was made to utilize cyclodextrin as a molecule recognition sensor in plant cells. The production of plant metabolites can vary widely depending on the culture condition, even among cell aggregates derived from the same source material. This heterogeneity in the cell culture metabolite profile is often exploited to screen for high producing cell lines in either solid or suspension cultures. Cho and Pedersen [62] developed a host-guest sensory system by modifying $\beta$-cyclodextrin. This proved to be both effective and convenient in the screening of suspension cultures of *E. californica* for benzophenanthridine alkaloids. The $\beta$-cyclodextrin was chemically modified to have a short tether (glycine) and a fluorophore (a dansyl moiety). The resulting dansyl-glycine-$\beta$-cyclodextrin is believed to have an exchangeable dansyl group that protrudes into the cyclodextrin cavity and can be relocated outside the cavity in the presence of the benzophenanthridine alkaloids as guest molecules. Therefore, the extent of complex formation leads to changes in the fluorescence intensity that can be correlated with guest molecule concentration. The sensitivity of this method is high enough to allow the use of a fluorescence plate reader. Thus, multiple samples can be assayed simultaneously in a few minutes. The extent of complex formation is known to dependent on atomic (van der Waals), thermodynamic (hydrogen bonding), and solvent (hydrophobic) forces between guest molecules and the essentially nonpolar environment of the cyclodextrin cavity. There have also been numerous efforts to use cyclodextrin as an artificial recognition molecule in sensors [63, 64]. Table 4 lists some of the derivatives that have received greatest attention.

**Table 4.** General structures of commonly used cyclodextrin (adapted from [65])




| Cyclodextrin | R | n |
|---|---|---|
| $\alpha$-Cyclodextrin | H | 4 |
| $\beta$-Cyclodextrin | H | 5 |
| $\gamma$-Cyclodextrin | H | 6 |
| Carboxymethyl-$\beta$-cyclodextrin | $CH_2CO_2H$ or H | 5 |
| Carboxymethyl-ethyl-$\beta$-cyclodextrin | $CH_2CO_2H$ or $CH_2CH_3$ or H | 5 |
| Diethyl-$\beta$-cyclodextrin | $CH_2CH_3$ or H | 5 |
| Dimethyl-$\beta$-cyclodextrin | $CH_3$ or H | 5 |
| Methyl-$\beta$-cyclodextrin | $CH_3$ or H | 5 |
| Random methyl-$\beta$-cyclodextrin | $CH_3$ or H | 5 |
| Glucosyl-$\beta$-cyclodextrin | Glucosyl or H | 5 |
| Maltosyl-$\beta$-cyclodextrin | Maltosyl or H | 5 |
| Hydroxyethyl-$\beta$-cyclodextrin | $CH_2CH_2OH$ or H | 5 |
| Hydroxypropyl-$\beta$-cyclodextrin | $CH_2CHOHCH_3$ or H | 5 |
| Sulfobutylether-$\beta$-cyclodextrin | $(CH_2)_4SO_3Na$ or H | 5 |

Recent efforts to modify cyclodextrin have been directed toward improving its safety in drug formulations while maintaining its high aqueous solubility for pharmaceutical applications. Recently, the two modified cyclodextrins, hydroxypropyl-$\beta$-cyclodextrins (HP-$\beta$-CDs) and sulfo-butylether-$\beta$-cyclodextrins (SBE-$\beta$-CDs) have received considerable attention due to their high water solubilities. Amorphous and non-crystalline HP-$\beta$-CDs have the advantage of greater aqueous solubility and a higher degree of hydroxypropyl substitution makes the drug binding poorer [66, 67]. It was found that partial methylation of the hydroxyls at the 2-, 3-, and/or 6-position of $\beta$-cyclodextrin generally leads to stronger complex formation but greater toxicity in drug formulations.

SBE-$\beta$-CDs are also highly water soluble and safe, but unlike HP-$\beta$-CDs, higher sulfobutyl group substitution often results in higher rather than lower drug binding [68].

The highly water-soluble 2-hydroxypropyl-$\beta$-cyclodextrin (2-HP-$\beta$-CD) is a commercially useful general complexing agent. Inclusion complexes of poorly water-soluble Naproxen with 2-HP-$\beta$-CD were useful to increase its solubility and dissolution rate, and resulted in an enhancement of bio-availability and minimized the gastrointestinal toxicity of the drug [69]. The water solubility of melatonin, which is an indole amide neurohormone, was also enhanced in a complex with 2-HP-$\beta$-CD [70].

Solubility of cyclodextrins in water can also be controlled by their degree of polymerization. Water insoluble polymers of cyclodextrin are preferred in some instances simply because they can stay in solid form and this leads to easy separation from the final product. In food processing, the browning effect due to the bioconversion of chlorogenic acid by the enzymatic action of polyphenol oxidase was reduced by using a cyclodextrin polymer, which removed the chlorogenic acid from the food materials [71]. The workers also found that the binding of chlorogenic acid by this cyclodextrin polymer was sensitive to water concentration because water acts as a molecular framework which keeps the chlorogenic acid and the cyclodextrin together as a complex. In another case, water-soluble cyclodextrin polymers were used to remove narigin, which is responsible for the bitter taste in citrus fruits [72].

Proper modification of the chemical moiety of cyclodextrin will be required to broaden the spectrum of application of cyclodextrins. However, achieving industrial significance using cyclodextrins in the plant cell system is dependent on cost, aqueous solubility, and safety.

## 5
## Integrated Bioprocessing with Aqueous Two-Phase Cultivation

### 5.1
### Aqueous Two-Phase Systems

Aqueous two-phase systems (ATPSs) are formed when two hydrophilic polymers such as dextran and polyethylene glycol are dissolved together above certain concentrations. Both phases are mainly composed of water enabling these systems to be used to separate and purify biomacromolecules and cells under nondenaturating conditions [73, 74]. Many polymers also form two-phase liquid-liquid systems when combined with suitable salts. Even though their main application is bioseparation, increasing attention has been focused upon their use in extractive bioconversions or in situ extractions [75]. The integration of extraction and production in a bioreactor increases the rate of product-inhibited processes or hydrophobic product formation. For product-inhibited processes, various methods have been reported to increase the production of a wide rage of chemicals and pharmaceuticals. However, the application of organic solvent usually introduces a level of toxicity to cells of diverse origin. The utilization of aqueous two-phase systems offers advantages in terms of designing an extractive bioconversion for cell cultivation because both phases are biocompatible. In this sense, extractive bioconversion using ATPSs is suitable for integrating cell culture and downstream processing for the enhanced production of an extracellular product. If the cells grow in one of the phases and the products are preferentially partitioned to the other phase, effective extractive in situ bioseparation is possible. For efficient in situ extraction, good cell growth in ATPS is necessary.

## 5.2
## Cultivation of Plant Cells in Aqueous Two-Phase Systems

### 5.2.1
### *Plant Cell Growth in Aqueous Two-Phase System*

Growth of microbial cells in ATPS was performed to examine the efficacy of extractive bioconversion [76]. In addition, animal cells such as hybridoma and Chinese hamster ovary cells were also cultured in these systems [77, 78]. Cultivation of plant cells in an aqueous two-phase polymer system was first reported by Hooker and Lee [79]. They showed that a two-phase system provided a viable medium for *Nicotiana tabacum* suspension culture. The productivity of secondary product was enhanced by immobilizing cells in one phase while collecting and withdrawing products from the other phase. Successful growth of plant cells in two-phase mixtures has also been carried out by other researchers [80, 81]. Along with suspension cells, hairy roots of *Tagetes patula* were grown in ATPSs to produce thiophene [82]. However, currently detailed analysis of plant cell culture in ATPSs is limited.

The aqueous two-phase system can be applied to plant cell suspension cultures as an in situ extraction method to enhance the production of hydrophobic secondary metabolites or extracellular proteins such as enzymes. Selective removal of the product during bioconversion using plant cells may also be possible in ATPS. Prior to obtaining phytochemicals with high yields using ATPS, however, reliable cultivation of plant cells in ATPS should be proven.

In our preliminary experiments, a 4.5% PEG 20,000 and 2.8% crude dextran system was made by mixing 15% of PEG 20,000 and 4% of crude dextran solution in the mass ratio of 1:2.33. This was selected for *Digitalis lanata* cell cultures on the basis of the distribution characteristics of cells and product. In this system, cells were totally partitioned in the bottom phase [83]. The growth pattern was investigated in shake flasks containing the selected aqueous two-phase polymer system and the results are shown in Fig. 14. For comparison, control cultures were performed in normal MS medium with respective cultivation in a medium containing 15% PEG 20,000 or 4% crude dextran, to clarify the influence of individual ATPS-forming polymer upon cell growth. Cell growth was significantly inhibited by adding only 15% of PEG 20,000. The length of the lag period was extended up to 4 days and slight growth was acknowledged. Crude dextran (4%) also lengthened the lag period, but the growth rate after the lag was much higher than that with PEG. However, in ATPS with both PEG and dextran, cell growth showed a similar pattern to that of the control. A toxic effect at high concentration of PEG was also observed in animal cell cultures [77], even though low concentrations of PEG were reported to stimulate cell growth [84]. The toxicity at the higher concentration levels may originate from changes in the physical properties of the culture medium. PEG may also contain some inhibitory compounds which could exert a toxic influence on the cells [76]. Since PEG is a well-known fusogen for various types of cells, the direct interaction of PEG with the cell surface may also affect cell growth. The molecular weight of PEG may be another factor that affects the plant cell growth. It has been shown

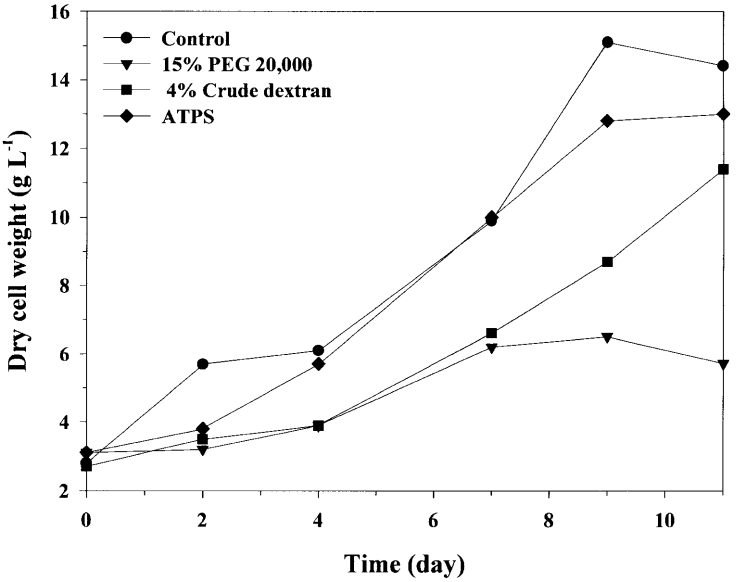



**Fig. 14.** Comparison of the cell growth in an individual polymer solution and ATPS. The composition of ATPS was 4.5% PEG 20,000 and 2.8% crude dextran which was a mixture of 15% PEG 20,000 and 2.8% crude dextran solution in the mass ratio of 1:2.33 [83]

that the growth rate and the stationary phase cell concentration decrease at lower PEG molecular weights in an aqueous two-phase cultivation of *Nicotiana tabacum* [79]. Although dextran was much less inhibitory once cell growth started, the concentration and molecular weight of dextran could similarly affect cell growth. The reason why it took a considerably longer time for cells to enter the exponential growth phase in ATPS and individual ATPS-forming polymer compared to control was possibly due to the fact that time was required for plant cells to adjust to the new environment. Ileva et al. [81] suggested that slow growth in an aqueous two-phase cultivation system might originate from the mass transfer limitations of the culture medium components.

It was interesting to observe that plant cells could grow successfully in ATPS despite the fact that their growth was inhibited in a solution of either of the polymer components of the ATPS system. We examined influences of ATPS and ATPS-forming polymers with respect to physical properties such as viscosity and the osmotic characteristics of the culture medium. Initially we measured the viscosity of all makeup solutions, which were water, modified MS medium, and 8% glucose solution in plain water without nutrients, and then measured their viscosities with PEG and dextran added alone and as an ATPS system. As shown in Fig. 15, it was found that the viscosity of the 4% crude dextran solution was very high, but that the 15% PEG solutions were much lower. The viscosity of any solution without polymer was about 1–2 cp. In a growth medium with dextran alone, the viscosity reached 350 cp. At this level, mixing adequate enough for cell culture was not possible. This may be the reason for the growth

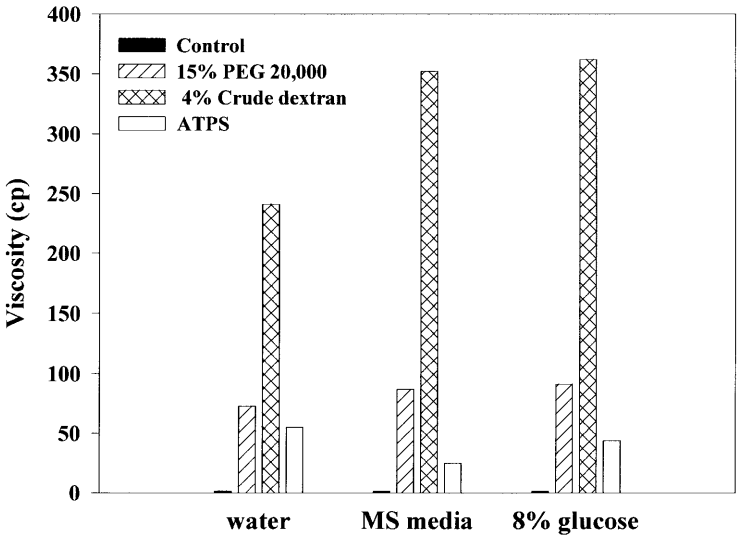



**Fig. 15.** Viscosity of each polymer solution and ATPS based on water, MS medium, and 8% glucose solution. The composition of ATPS was 4.5% PEG 20,000/2.8% crude dextran [83]

inhibition shown in Fig. 14. When both polymer solutions were mixed to form ATPS, the viscosity reduced because of emulsion formation, which probably enhanced the oxygen transfer that is required for cell growth. The reasonable cell growth observed in ATPS could probably be explained by this reduction in medium viscosity. Hooker and Lee [79] pointed out the importance of the miscibility of the two polymer phases. Higher phase miscibility may cause less resistance to nutrient mass transfer, including oxygen, thus facilitating growth. As the cells were completely partitioned in the dextran phase, direct contact with PEG could be prevented. Cell growth in ATPS seemed to be much better than that in either phase-forming polymer.

Dextran and especially PEG were found to have a strong concentration-dependent effect on the osmotic characteristics of the animal cell culture medium [77]. Therefore, the effect of ATPS-forming polymers on osmotic pressure in a plant cell culture medium was examined [83]. As shown in Fig. 16, the addition of 15% PEG into the MS medium produced an osmotic pressure of over 640 mOsm $kg^{-1}$ while 4% crude dextran gave an osmotic pressure of 358 mOsm $kg^{-1}$. In ATPS, 395 mOsm $kg^{-1}$ was observed (301 mOsm $kg^{-1}$ in normal MS medium). The 8% glucose solution showed a much higher osmotic pressure due to the presence the sugar. Osmolality is an important variable in animal cell culture, but this is not the case in a plant cell suspension culture. Since plant cells have cell wall, this range of osmolality is endurable. High osmotic pressure usually reduced plant cell size without having an apparent effect on the growth rate.

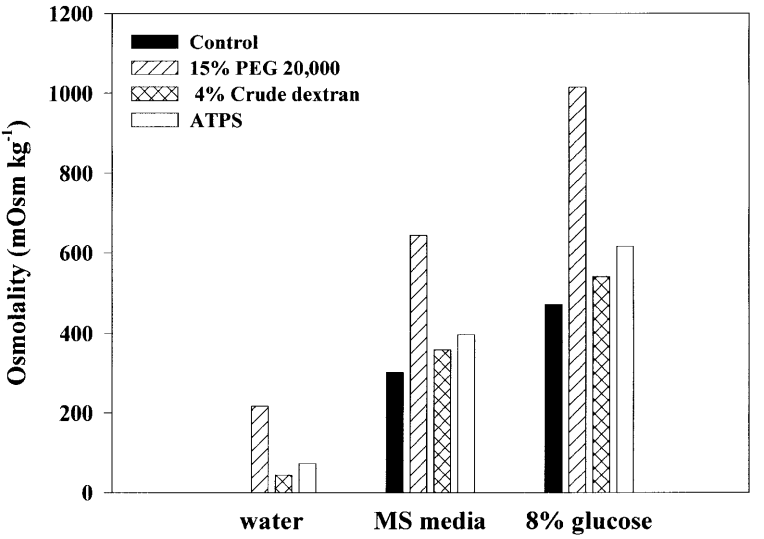



**Fig. 16.** Osmolality of each polymer solution and ATPS based on water, MS medium, and 8% glucose solution

### 5.2.2
### *Aqueous Two-Phase Cultivation of Plant Cells in a Bioreactor*

In order to confirm the scale-up possibility of plant cell growth in ATPS, bioreactor cultivation was performed. Cell growth in a 4.5% PEG 20,000/2.8% crude dextran ATPS was monitored in a 5-l stirred tank bioreactor with a 2-l working volume [83]. The growth pattern was similar to that of shake flasks. Aqueous two-phase cultivation of *D. lanata* suspension cells was made possible in bioreactors and the final cell mass was slightly lower than that of the control (Fig. 17). During the cultivation in ATPS, the ratio of fresh cell weigh to dry cell weight was maintained at a low value compared to the control, due to the high osmolality of the two-phase medium. The viscosity of the aqueous two-phase cultures was maintained between 25 cp and 35 cp. However, in a control culture, it increased from 6 cp at the beginning of cultivation to 25 cp at the end.

It was also found that plant cell suspension culture in an aqueous two-phase polymer system was feasible in shake flasks as well as in bioreactors. This was made possible by lowering the viscosity using ATPS, which promoted oxygen transfer. However, the cell growth achieved in ATPS fell somewhat short of reaching the growth level of the control culture. Therefore, factors which enhance the cell growth in ATPS up to or more than the control level should make this system more valuable. In addition to cell growth, the in situ production of phytochemicals should be studied. Since the production of digoxin by digitoxin biotransformation was enhanced by in situ adsorption in *D. lanata* cell cultures [85], it is expected that an application of ATPS with an adequate integrated bioprocess possesses the potential to enhance the digoxin production. This system

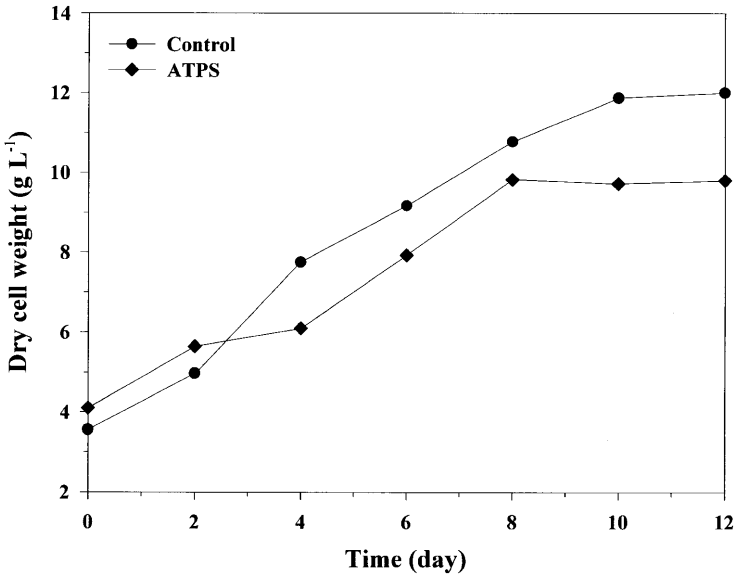



**Fig. 17.** Aqueous two-phase cultivation in a plant cell bioreactor

can also be used for the production of secretory enzymes or recombinant proteins in plant cell cultures.

### 5.3
### In situ Extraction Using Aqueous Two-Phase Cultivation of Plant Cells

A combination of an extractive step with plant cell culture for product separation has been known be a feasible technology for enhancing the productivity as well as reducing downstream processing costs. In terms of the extraction itself, a great deal of experience has been accumulated. For the biotechnological application of in situ extraction, using aqueous two-phase systems can provide many advantages over the utilization of organic solvents. Since aqueous two-phase systems have high water content and a low interfacial surface tension, they are regarded as biocompatible [86]. Tjerneld and Johansson summarized the different kinds of ATPSs in terms of their potential use in biotechnology [87]. Although ATPS was developed initially for the separation and purification of biomolecules and cells, a large number of applications have recently been identified [88]. Increasing attention has been focused on the use of ATPSs in bioconversion and de novo production where enzymes, microbial cells, animal cells, or plant cells are used. The adoption of solvent extraction in a production process introduces the possibility of inhibition, because organic solvents are generally toxic to cells, which favors the use of a wholly water-borne system for in situ extraction [75]. If the cells grow in one of the phases and the products are preferentially partitioned to the other phase, an effective in situ extraction is possible. Until now, few results have been published on the use of these systems with plant cell and tissue culture. Cell suspension cultures of *Nicotiana ta-*

*bacum* [79–81], *Lavandula vera* [81], and *Digitalis lanata* [83] were cultivated in ATPSs. Hairy roots of *Tagetes patula* were also grown in ATPSs [82]. However, applications of in situ extraction with cell cultivation are limited. When *Tagetes patula* hairy roots were grown in ATPSs to produce thiophene, the excretion of thiophene in the bioreactor was about ten times higher than that in the control [82]. Their growth rate was lower in ATPSs than in standard medium. The hairy roots were confined completely to the bottom phase, while the product had a slight preference for the top phase. Another paper regarding the use ATPSs described the production of enzymes. The growth of *Nicotiana tabacum* in an ATPS as well as the biosynthesis, secretion, and partitioning of acid and alkaline phosphomonoesterases were reported [80]. The yield of acid and alkaline extracellular phosphomonoesterases were 18 and 10 times higher, respectively. Partitioning took place mainly in the bottom phase with 4.5 and 3.5 times higher specific activity, respectively.

The above two reports show promising results concerning the use of ATPSs in plant cell and tissue culture for the production of secondary metabolites and protein products such as extracellular enzymes and recombinant proteins. Even though aqueous two-phase systems have the potential to be used for plant cell cultures, several problems remain to be solved. First, plant cell growth in ATPS should be optimized, and second, an adequate permeabilizing technique should be developed because most of the secondary products are stored in cells. Third, integration of various processes including elicitation, growth promotion, and the addition of oxygen vectors to enhance oxygen transfer should be investigated.

## 6 Concluding Remarks

Integrated bioprocesses can be used to enhance the production of valuable metabolites from plant cell cultures. The in situ removal of product during cell cultivation facilitates the rapid recovery of volatile and unstable phytochemicals, avoids problems of cell toxicity and end-product inhibition, and enhances product secretion. In situ extraction, in situ adsorption, the utilization of cyclodextrin, and the application of aqueous two-phase systems have been proposed for the integration of cell growth and product recovery in a bioreactor. The simultaneous combination of elicitation, immobilization, permeabilization, and in situ recovery can promote this method of plant cell culture as a feasible method to produce various natural products including proteins.

Since most organic solvents are harmful to plant cell growth, the selection of an adequate lipophilic phase is essential for successful in situ extraction. Elicitation in conjunction with in situ extraction could alter the pattern of secondary metabolite production and enhance the product yield.

In terms of in situ adsorption, although none of the solid adsorbents for nonaqueous phase tested to date are entirely satisfactory for the selective separation of secondary metabolites formed by plant cells, the result obtained so far have been encouraging enough to stimulate the search for more selective solid adsorbent material. The most important advantages of the two-phase culture techniques are that it increases productivity by removing the feedback regula-

tion induced by secondary metabolites and nonspecific inhibitors, and makes the continuous bioreactor possible.

The addition of cyclodextrin, or modified cyclodextrin, offers another possible potential for the enhanced production of hydrophobic, toxic, volatile, or unstable products from plant cell cultures. As an extractive production system, product formation and separation can occur simultaneously in the presence of cyclodextrin, which results in the enhancement of product level.

Aqueous two-phase cultivation of plant cells was found to be possible when the culture conditions were optimized. The selection of proper polymers and the optimization of their concentrations are needed not only for cell growth, but also for the desirable product partition. Promising examples of the use of aqueous two-phase systems for in situ extraction have been reported recently. However, more detailed and broader studies are necessary to utilize fully the potential of aqueous two-phase systems in plant cell culture. Finally, we believe that the development of fully integrated processes for plant cell cultures will improve the production efficiency of different kinds of secondary metabolites, enzymes, and recombinant proteins.

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## References

1. Fett-Neto AG, DiCosmo F (1996) Production of paclitaxel and related taxoids in cell cultures of *Taxus cuspidata*: perspectives for industrial application. In: DiCosmo F, Misawa M (eds), Plant cell culture secondary metabolism toward industrial application. CRC Press, Boca Raton, p 139
2. Miele L (1997) Trends Biotechnol 15:45
3. Kurata H, Kawai A, Seki M, Furusaki S (1994) J Ferment Bioeng 78:117
4. Williams RD, Chauret N, Bedard C, Archambault J (1992) Biotechnol Bioeng 40:971
5. Asada M, Shuler M (1989) Appl Microbiol Biotechnol 30:475
6. Brodelius P, Pedersen H (1993) Trends Biotechnol 11:30
7. Bisson W, Beiderbeck R, Reichling J (1983) Planta Med 47:164
8. Maisch R, Knoop B, Beiderbeck R (1986) Z Naturforsch 41c:1041
9. Kim DJ, Chang HN (1990) Biotechnol Bioeng 36:460
10. Byun SY, Pedersen H, Chin CK (1990) Phytochemistry 29:3135
11. Collinge MA, Brodelius PE (1989) Phytochemistry 28:1101
12. Ten Hoopen HJG, Gulik WM, Meijer JJ (1990) Proceedings of the VIIth International Congress on Plant Tissue and Cell Culture. Amsterdam, p 673
13. Byun SY, Pedersen H (1994) Biotechnol Bioeng 44:14
14. Skinner NE, Walton NJ, Robins RJ, Rhodes MJC (1987) Phytochemistry 26:721
15. Payne GF, Payne NN, Shuler ML, Asada M (1988) Biotechnol Lett 10:187
16. Payne GF, Shuler ML (1988) Biotechnol Bioeng 31:922
17. Dörnenburg H, Knorr D (1995) Enzyme Microb Technol 17:674
18. Choi JW, Yoo DI, Lee WH, Pedersen H (1996) J Ferment Bioeng 81:47
19. Choi JW (1990) PhD thesis, Rutgers University
20. Choi JW (1992) Kor J Chem Eng 9:128
21. Nigam SC, Wang HY (1986) Mathematical modeling of bioproduct adsorption using immobilized affinity adsorbents. In: Asenjo JA, Hong J (eds) Separation, recovery and purification in biotechnology. American Chemical Society, Washington DC, USA, p 153

22. Nigam SC, Siahpush AR, Wang HY (1990) AIChE J 36:1239
23. Sim SJ, Chang HM, Liu JR, Jung KH (1994) J Ferment Bioeng 78:229
24. Knoop B, Beiderbeck R (1983) Z Naturforsch 38c:484
25. Beiderbeck R, Knoop B (1984) Z Naturforsch 39c:45
26. Knuth ME, Sahai OP (1991) US Pat 5,068,184
27. Becker H, Reichling J, Bisson W, Herold S (1984) 3rd European Congress on Biotechnology, Dechema, vol 1, p 209
28. Cormier F, Do CB (1988) Selection of monoterpene producing *Mentha piperita* cell lines. In: Schreier P (ed) Bioflavour '87. Walter de Gruyter, Berlin, p 357
29. Strobel J, Hieke M, Gröger D (1991) Plant Cell Tiss Org Cult 24:207
30. Berlin J, Witte L (1988) Phytochemistry 27:127
31. Kim DI, Hong HJ, Lee JE, Choi YS (1997) Proceedings of Asia-Pacific Biochemical Engineering Conference '97, vol 1, Beijing, p 516
32. Forche E, Schubert W, Kohl W, Höfle G (1984) 3rd European Congress on Biotechnology, vol 1, Dechema, p 189
33. Parr AJ, Robins RJ, Rhodes MJC (1987) Release of secondary metabolites by plant cell cultures. In: Webb C, Mavituna F (eds) Plant and animal cells: process possibilities. Ellis Horwood, Chichester, p 229
34. Robison RC, Cha DY (1985) Biotechnol Prog 1:18
35. Robins RJ, Rhodes MJ (1986) Appl Microbiol Biotechnol 24:35
36. Garcia AA (1991) Biotechnol Prog 7:33
37. Payne GF, Shuler ML (1985) Biotechnol Bioeng Symp 15:633
38. Villadsen JV, Michelsen M (1978) Solution of differential equation models by polynomial approximation. Prentice-Hall, Englewood Cliffs, USA, p 141
39. Metzler CM, Elfring GL, McEwen AJ (1974) Biometrics 30:562
40. Choi JW, Kim YK, Park HK, Lee WH, Kim DI (1999) Biotechnol Bioprocess Eng 4:281
41. Lee JE, Lee SY, Kim DI (1999) Biotechnol Bioprocess Eng 4:32
42. Szejtli J (1988) Cyclodextrin technology. Kluwer Academic Publishers, The Netherlands
43. Haggin J (1992) Chem Eng News 70:25
44. van Uden W, Woewrdenbag HJ, Pras N (1994) Plant Cell Tiss Org Cult 38:103
45. Loftsson T, Brewster M (1996) J Pharm Sci 85:1017
46. Rajewski RA, Stella VJ (1996) J Pharm Sci 85:1142
47. Irie T, Uekama K, Hirayama F (1994) Drug Targeting Delivery 3:411
48. Elisabeth PCK, Patricia GY, Genevieve WS, Faan WB, William JJ, Michal CP, George HR (1995) J Biochem Mol Biol 270:17,250
49. Ji JR, Yoo IJ, Park WM, Jeon KH, Kim CJ, Lim SB (1997) Korean J Anim Sci 39:599
50. Antonio M, Maria A, Paola M, Marco A, Michael C, Olivieri R, (1995) Arch Microbiol 164:290
51. Han SM (1977) Biomed Chromatogr 11:259
52. Tahara S, Shinagawa K, Minato K (1999) J Chromatogr 848:465
53. Froamming K, Szejtli J (1994) Cyclodextrin in pharmacy. Kluwer Academic Publishers, Dordrecht
54. Ramachadra RS, Ravishanker GA (1999) Process Biochem 35:341
55. Hopp R (1993) Some highlights of H&R research: a review of nearly 120 years of research at Haarmann & Reimer. In: Hopp R, Mori K (eds) Recent developments in flavour and fragrance chemistry. VCH Publishers, Weinheim, Germany, p 14
56. Woerdenbag H, Pras N, Frijlink H, Lerk C, Malingre TM (1990) Phytochemistry 29:1551
57. Lee JE, Hong HJ, Kim DI (1995) Theories and applications of chemical engineering, vol 1. Korean Institute of Chemical Engineers, Seoul, Korea, p 479
58. Ramachandra RS (1998) PhD thesis, University of Mysore, India
59. Lim CH, Park SC, Shin MK, Cho GH (1996) Korean J Biotechnol Bioeng 11:411
60. Graf S, Knorr D (1993) Multiple shoot cultures of *Mentha canadensis* for biotechnological production of flavours. In: Schreier P, Winterhalter P (eds) Progress in flavour precursor studies, vol 4. Carol Stream: Allured Publishers, p 471
61. Park SC, Cho GH (1996) Korean J Biotechnol Bioeng 11:411

62. Cho GH, Pedersen H (1998) Biotechnol Tech 12:833
63. Litwiler KS, Cattena GC, Bright FV (1990) Chimica Acta 237:485
64. Ueno A, Suzuki I, Osa T (1990) Anal Chem 62:2461
65. Stella VJ, Rajewski RA (1997) Pharm Res 14:556
66. Muller BW, Bbrauns U (1986) J Pharm Sci 75:571
67. Pitha J (1987) J Controlled Release 6:309
68. Zia V, Rajewski R, Bornancini ER, Luna EA, Stella VJ (1997) J Pharm Sci 86:220
69. Lee BJ, Lee JR (1995) Arch Pharm Res 18:22
70. Lee BJ, Choi HG, Kim CK, Keith AP, James WA, Robert LS (1997) Arch Pharm Res 20:560
71. Irwin PL, Brouillette JN, Giampa AJ, Hicks KB, Gehring AG, Tu SI (1998) Carbohydr Res 322:76
72. Woo GJ, Ha SM (1997) Korean J Food Sci Technol 29:302
73. Kula MR (1990) Bioseparation 1:181
74. Albertsson P, Johansson G, Tjerneld F (1990) Aqueous two-phase separations. In: Asenjo JA (ed) Separation processes in biotechnology. Marcel Dekker, New York, p 287
75. Kaul R, Mattiasson B (1991) Extractive bioconversions in aqueous two-phase systems. In: Mattiasson B, Holst O (eds) Extractive bioconversion. Marcel Dekker, New York, p 173
76. Kaul R, Mattiasson B (1986) Appl Microbiol Biotechnol 24:259
77. Zijlstra GM, de Gooijer CD, van der Pol LA, Tramper J (1996) Enzyme Microb Technol 19:2
78. Zijlstra GM, Michielsen MJF, de Gooijer CD, van der Pol LA, Tramper J (1996) Biotechnol Prog 12:363
79. Hooker BS, Lee JM (1990) Plant Cell Rep 8:546
80. Ileva MP, Bakalova A, Mihneva M, Pavlov A, Dolapchiev L (1996) Biotechnol Bioeng 51:488
81. Ileva M, Kojuharova A, Pavlov A, Mihneva M, Shterev I (1995) Biotechnol Biotechnol Eq 9:71
82. Buitelaar RM, Leenen EJTM, Tramper J (1992) Biocatalysis 6:73
83. Choi, YS, Lee SY, Kim DI (1999) J Microbiol Biotechnol 9:589
84. Shintani Y, Iwamoto K, Kitano K (1988) Appl Microbiol Biotechnol 27:533
85. Hong HJ, Lee JE, Ahn JE, Kim DI (1998) J Microbiol Biotechnol 8:478
86. Mattiasson B, Ling TGI (1987) Extraction in aqueous two-phase systems for biotechnology. In: Verrall MS, Hudson MJ (eds) Bioseparation for biotechnology. Ellis Horwood, Chichester, p 270
87. Tjerneld F, Johansson G (1990) Bioseparation 1:255
88. Walter H, Johansson G (1994) Methods in enzymology, vol 228. Aqueous two-phase systems. Academic Press, San Diego

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# Genetic Modification of Plant Secondary Metabolite Pathways Using Transcriptional Regulators

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Plant secondary metabolism is the source of many natural products with diverse applications, including pharmaceuticals, food colors, dyes and fragrances. Functions in plants include attraction of pollinating insects and protection against pests and pathogens. An important regulatory step in secondary metabolism is transcription of the biosynthetic genes. The aim of this chapter is to discuss results and opportunities concerning modification of secondary metabolism using transcriptional regulators. The transcriptional regulation of two well-studied secondary pathways, the phenylpropanoid pathway and its flavonoid branch, and the terpenoid indole alkaloid biosynthetic pathway, are reviewed. Some examples of successful engineering of these pathways via transcriptional regulators are discussed.

**Keywords.** Anthocyanins, AP2-domain, Jasmonate, Terpenoid indole alkaloids, Transcription factor



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## Abbreviations

| | |
|---|---|
| AP2 | APETALA2 |
| AP2/ERF | APETALA2/ethylene-responsive factor |
| bHLH | basic helix-loop-helix |
| bZIP | basic leucine zipper |
| Ca | calcium |
| CaMV | cauliflower mosaic virus |
| cDNA | complementary DNA |
| JA | jasmonic acid |
| JERE | jasmonate-and elicitor-responsive element |
| MADS | MCM1-AGAMOUS-DEFICIENS-SRF |
| MAPK | mitogen-activated protein kinase |
| MeJA | methyl jasmonate |
| 4-mT | 4-methyltryptophan |
| ODA | octadecanoid |
| ORCA | octadecanoid-responsive *Catharanthus* AP2-domain |
| STR | strictosidine synthase |
| TDC | tryptophan decarboxylase |
| T-DNA | transferred DNA |
| TIA | terpenoid indole alkaloid |
| UV | ultraviolet |
| VP1 | VIVIPAROUS-1 |

## 1 Introduction

Prospects to modify secondary metabolite production in plants or plant cells via genetic engineering of a single or a few enzymatic steps have been reviewed previously [1–3]. The aim of this chapter is to discuss opportunities to modify secondary metabolism using transcriptional regulators. Transcriptional regulation is largely mediated by sequence-specific DNA-binding proteins that recognize *cis*-acting DNA elements located in the promoter and enhancer regions of the corresponding genes. In general, these *cis*-acting elements are concentrated in a relatively small region of a few hundred to a few thousand nucleotides upstream of the transcriptional start site. In most genes, this start site is determined by the presence of a TATA box around 30 bp upstream. On the TATA box the basal transcription machinery is assembled. Together with RNA polymerase II and a large number of other proteins, the preinitiation complex is formed. During initiation of transcription, the preinitiation complex shifts from the closed to the open configuration. Transcription factors regulate gene transcription in response to developmental, tissue-specific or environmental signals by binding with their so-called 'DNA-binding domains' to specific DNA sequences in the gene promoters. Transcription factors may modify the rate of transcription initiation by controlling the rate of recruitment of components of the preinititation complex. Alternatively, they can regulate the rate of transition of the preinitiation complex from the closed to the open configuration, or via other

mechanisms (reviewed in [4]). Transcription factors may affect these processes by direct interactions between their so-called 'activation-domains' and components of the basal transcription machinery, or by indirect interactions via adapter or co-activator proteins (reviewed in [5]).

Depending on the epigenetic make-up of the target cell, single transcription factors can switch on complex pathways involving numerous target genes. A notable example is muscle differentiation in animals, where either one of a set of myogenic bHLH transcription factors (MyoD, myogenin, Myf5, MRF4) in combination with the MADS-domain transcription factor MEF2 induces muscle cell differentiation and switches on numerous muscle-specific genes in skin fibroblasts and certain other cell types [6]. Other examples include single homeodomain transcription factors in the fruit fly, which regulate complex pathways resulting in the determination of segment identity [7, 8]. In *Arabidopsis thaliana*, overexpression of the transcription factor CBF-1 results in coordinate upregulation of a set of cold-regulated genes, and in increased freezing tolerance [9]. These examples illustrate that single transcription factors can act as master regulators of complex gene expression programs, and can control the expression of a large number of target genes in a coordinate fashion. A general characteristic of secondary metabolic pathways is that the expression of the structural genes is coordinately regulated depending on cell type or in response to environmental stimuli. This coordinate control is most likely due to master regulators, and one can envisage that secondary metabolism can be modified by engineering the activity of such master switches. The following sections will review some aspects of the transcriptional regulation of two well-studied secondary metabolic pathways, the phenylpropanoid pathway and its flavonoid branch, and the terpenoid indole alkaloid biosynthetic pathway. Some examples of successful engineering of these pathways via transcriptional master regulators will be discussed.

## 2 Regulation of the Phenylpropanoid and Flavonoid Pathways

The phenylpropanoid pathway is the source of a large number of compounds that are derived from the amino acid phenylalanine (Fig. 1). The flavonoid branch is responsible for the production of anthocyanin pigments, UV-absorbing flavones and flavonols, and antimicrobial phytoalexins. Other branches produce lignin precursors and soluble phenolics such as the signaling compound salicylic acid. A large number of structural genes have been cloned from many plant species [10]. The best-studied aspect of transcriptional regulation regards the formation of the anthocyanin pigments, due to the fact that they provide a convenient visible marker. Another well-studied aspect is the stress- and pathogen-induced biosynthesis of phytoalexins. Both classes of end products share a large part of the biosynthetic pathway. The structural flavonoid genes are subject to different regulatory mechanisms within a single plant depending on the cell type and environmental conditions.

Tissue-specific expression of the flavonoid structural genes is controlled by a combination of two distinct transcription factor species, one of which has

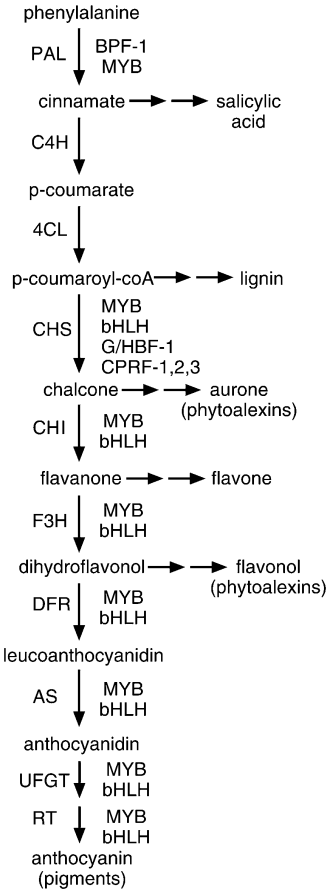


**Fig. 1.** Simplified diagram of the phenylpropanoid and flavonoid biosynthetic pathways. Enzymes that catalyze the reactions are placed on the left-hand side, and transcription factors on the right-hand side of the arrows. Both transcription factors for which their control over the enzymatic steps has been genetically proven, as well as transcription factors that have been shown to interact with promoters of the structural genes, are shown. *PAL* Phenylalanine ammonia lyase; *C4H* cinnamate 4-hydroxylase; *4CL* 4-coumaroyl-coenzyme A ligase; *CHS* chalcone synthase; *CHI* chalcone-flavanone isomerase; *F3H* flavanone 3$\beta$-hydroxylase; *DFR* dihydroflavonol 4-reductase; *AS* anthocyanin synthase; *UFGT* UDP glucose-flavonol glucosyl transferase; *RT* anthocyanin rhamnosyl transferase

homology to the protein encoded by the vertebrate proto-oncogene c-*Myb*, and the other to the vertebrate basic helix-loop-helix (bHLH) protein encoded by the proto-oncogene c-*Myc*. These transcription factors bind to specific sequences in the promoters of the target genes [11–13]. The DNA-binding domain of plant MYB proteins consists of two, or for some one or three, imperfect repeats [14, 15]. The DNA-binding specificity of plant MYB proteins varies considerably [14]. The bHLH proteins recognize variants of the sequence CANNTG. In particular, the G-box (CACGTG) has been reported to interact

with bHLH factors [16, 17]. The G-box is also a target for basic leucine zipper (bZIP) transcription factors [18]. Plant MYB and bHLH proteins can physically interact with each other [19]. In maize, the entire set of genes encoding enzymes in anthocyanin biosynthesis is thought to be regulated coordinately by the bHLH protein-encoding gene *R* and the *Myb* gene *C1* in the aleurone (epidermal layer of the kernel endosperm), and by homologous genes in other parts of the plant [10, 20] (Fig. 1). In dicotyledonous plants, anthocyanin control appears to be more complex. Although all the structural genes in the anthocyanin branch of the pathway are coordinately regulated during flower development, the earlier and later parts of the biosynthetic pathway are thought to be controlled independently [20]. Nevertheless, introduction of the maize *R* and *C1* regulators in *Arabidopsis* intensifies pigmentation in normally pigmented tissues and induces pigmentation in plant tissues that are normally unpigmented [21]. Overexpression of the maize *Lc* gene, which encodes a bHLH regulatory protein, in petunia upregulated the whole flavonoid biosynthetic pathway starting from *Chs* and including the earlier and later genes, resulting, among others, in intensely pigmented leaves [22].

The expression of the MYB and bHLH protein-encoding genes coincides with that of the structural genes that they regulate [20, 23]. The mere presence of a MYB and a bHLH protein in a cell appears to switch on the expression of the anthocyanin structural genes. Although the DNA-binding affinity of an *Antirrhinum* MYB protein was negatively affected by phosphorylation [12], evidence that the activity of MYB and bHLH proteins is regulated by cell-type-specific mechanisms involving protein modification in vivo is lacking. Additional regulators of anthocyanin biosynthesis have been scarcely identified. Notable examples are the petunia *AN11* gene [24] and the *TTG1* gene from *Arabidopsis* [25]. The expression of the *Dfr* anthocyanin structural gene in the petunia *an11* mutant can be rescued by expression of the MYB protein AN2, indicating that the AN11 protein acts upstream of MYB. Since the *An*2 mRNA level is normal in the *an11* mutant, it has been suggested that the AN11 protein regulates MYB protein activity [24]. In contrast to the *Myb* gene *An*2, *An*11 is ubiquitously expressed. The *Arabidopsis ttg1* mutant has many aberrant phenotypes, including lack of anthocyanin biosynthesis. The *ttg1* mutant phenotype can be rescued by expression of the maize *R* gene [21], indicating that the TTG1 protein regulates the activity of a bHLH-type protein. AN11 and TTG1 proteins are highly similar, and both contain four WD repeat motifs, which are also found in the $\beta$ subunits of heterotrimeric G proteins and a number of other regulatory proteins. Given their high similarity, it seems unlikely that AN11 and TTG1 have different functions, and they probably regulate the activity of MYB-bHLH complexes. Another example of an additional regulator of anthocyanin biosynthesis is the VP1 protein. The maize *viviparous1* mutant does not produce anthocyanin in seeds, while other plant parts are normally pigmented. The *vp1* mutation also affects seed maturation, resulting in shoots and roots that emerge from the seeds while the kernel is still attached to the ear. Certain *vp1* alleles prevent anthocyanin biosynthesis but produce normal, nonviviparous seed, suggesting that control of the anthocyanin pathway is at least partially separable from regulation of embryo maturation. The *vp1-R* mutant

fails to express the *C1* gene in developing seed tissues [26], and the VP1 protein can transactivate a reporter gene driven by the *C1* promoter [27] by specifically binding to the Sph sequence [28]. Therefore, VP1 is a transcription factor that acts upstream of the MYB protein *C1* by regulating its expression via direct binding to the promoter.

The fact that so few mutants affected in regulation of MYB and bHLH activity have been found in plants is surprising in view of the multitude of protein modifications and protein–protein interactions reported for their mammalian counterparts. Mammalian MYC is active only when complexed with the bHLH protein MAX [29]. The potential of MYC to activate transcription is suppressed by association with p107, an Rb-related tumor suppressor [30]. Phosphorylation stimulates transactivation by MYC [31]. Phosphorylation [31, 32] or reversible oxidation [33] of MYB reduces its DNA-binding affinity. The interaction of MYB with the general co-activator CREB binding protein (CBP) is required for transactivation [34, 35]. It can be expected that similar interactions and modifications play a role in the regulation of the activity of plant bHLH and MYC proteins. The fact that no mutants have yet been found in these regulatory processes may be due to the fact that the genes involved are redundant or absolutely essential.

Many phenylpropanoid compounds that are constitutively present in certain cell types and/or during development can be produced in other tissues in response to various stress-related signals [36]. Bean *Pal* and *Chs* genes are rapidly and coordinately induced by elicitors [36]. UV light induces the transcription of the parsley *Chs* gene [37]. In the promoters of phenylpropanoid biosynthetic genes from various plant species common sequence motifs can be identified. The H-box [(T/A)CT(C/A)ACCTA(C/A)C(C/A)] and box P [CCA(C/A)C(A/T)AAC(C/T)CC] are present in the promoters of *Chs*, *Pal* and *4-cl* genes in different plant species, and have been associated with regulation of gene activation by stress stimuli such as UV light or elicitors [37–40]. In the *Chs* gene from parsley [37, 41] and *Arabidopsis* [42], a light-responsive unit (LRU) has been identified, which is necessary and sufficient to confer gene expression upon UV-B and UV-A/blue light illumination. The LRU is composed of a H-box, also called a MYB recognition element (MRE) and an ACGT element. In the parsley *Chs* gene the ACGT element forms part of a G-box (CACGTG), which is a conserved element in plant promoters [18]. The ACGT element has been found to interact with the parsley bZIP class proteins CPRF-1, 2 and 3 [41]. CPRF-1 transcripts are induced by UV light with much faster kinetics than *Chs* mRNA, suggesting that CPRF-1 is responsible for UV-responsive expression of the *Chs* gene. The *Arabidopsis* bZIP protein HY5 also interacts with the G-box in the LRU [43]. The H- (or MRE) box has been found to interact with DNA-binding proteins of the MYB class [44, 45]. The parsley MYB protein *Pc*MYB1 transactivates expression of an MRE-containing reporter construct [45]. In contrast to *Chs* (and the *CPRF-1* gene), *PcMyb*1 expression is not light-regulated. In addition, the H- (or MRE) and G-boxes in the bean *Chs* gene were shown to interact with a bZIP class protein with relaxed DNA-binding specificity called G/HBF-1 [46]. While G/HBF-1 transcript and protein levels do not increase during the induction of phenylpropanoid biosynthetic genes, the DNA-binding affinity of the G/HBF-1

protein is enhanced by elicitor-induced phosphorylation, suggesting that G/HBF-1 is responsible for elicitor-responsive expression of the bean *Chs* gene. However, there are no in vivo expression studies that prove that the H- and G-boxes are involved in elicitor-responsive expression of the bean *Chs* gene. The P-box in the parsley *Pal* gene was shown to interact with BPF-1, a protein with some similarity to MYB transcription factors [47]. Expression of the gene encoding BPF-1 is induced by fungal elicitor with slightly faster kinetics than those of *Pal* expression. More recent studies have shown that box P is a relatively low-affinity binding site for BPF-1 [48], indicating that the in vivo relevance of the box P/BPF-1 interaction is questionable.

The functional identification of these transcription factors is based on more or less specific recognition of stress-responsive elements in the promoters of the structural genes, and sometimes their stress-induced expression. Definitive proof of their function awaits (reverse) genetic evidence. Although the conservation of the binding sites in different structural genes suggests that some of these stress-related transcription factors coordinate the expression of several genes, experimental evidence is lacking. It is not known how stress-induced expression of phenylpropanoid biosynthetic genes ties in with their tissue-specific control by MYB and bHLH proteins. Since a MYB and a bHLH protein can induce pigmentation in otherwise unpigmented tissues without stress signals, it appears that they overrule stress-signaling pathways. On the other hand, the structural genes can be induced by stress signals in tissues that appear to lack the appropriate tissue-specific MYB and/or bHLH proteins. Whether there is crosstalk between tissue-specific MYB/bHLH regulation and stress-induced signaling remains an open question.

## 3 Modification of Flavonoid Metabolism Using Transcription Factors

*Myb* and bHLH protein-encoding genes have been ectopically expressed in the plant species from which they originated, as well as in heterologous plant species. All studies described here used the cauliflower mosaic virus (CaMV) 35S RNA promoter, which is highly active in most tissues of most plant species, to express the transcription factors.

Introduction of the *Delila* (*del*) gene from *Antirrhinum*, encoding a bHLH transcription factor, in tomato strongly increased pigmentation in vegetative tissues [49]. In tobacco, *del* only intensified pigmentation of flowers, whereas in *Arabidopsis*, *del* had no phenotypic effects. This indicates that DELILA is active in some, but not all, heterologous plant species, and recognizes its orthologous target genes. Overexpression of the maize *Lc* gene encoding a bHLH regulatory protein in petunia upregulated the whole flavonoid biosynthetic pathway starting from *Chs* and including the earlier and later genes, resulting among others in intensely pigmented leaves [22]. The expression of the general phenylpropanoid genes *Pal* and *C4h* was not affected by *Lc* overexpression, indicating that *Lc* only regulates structural genes in the flavonoid branch. Ectopic expression of the bHLH transcription factors DELILA and *Lc* led to increased pigmentation only in tissues that are normally pigmented, or that can become pigmented

under stress conditions. The ectopically expressed bHLH proteins probably rely on endogenous MYB proteins to activate their target genes.

Introduction of the maize *R* and *C1* regulators in *Arabidopsis* intensified pigmentation in normally pigmented tissues and induced pigmentation in plant tissues that are normally unpigmented [21]. In maize cell suspension, ectopic expression of *C1* and *R* led to the accumulation of anthocyanins and a number of other related 3-hydroxyflavonoids [50]. In addition, six anthocyanin structural genes that are targets for *C1*/*R* were expressed at high levels in the transgenic cell line.

These experiments demonstrate that the ectopic expression of transcription factors is a viable method for engineering secondary metabolism in plants and plant cells.

## 4 Jasmonates as Stress Signals in Secondary Metabolism

Jasmonic acid (JA) and its volatile derivative methyl jasmonate (MeJA), collectively called jasmonates, play key regulatory roles in stress-induced secondary metabolism in plants.

Jasmonates are fatty acid derivatives with a 12-carbon backbone, which are synthesized from 18-carbon intermediates via the so-called octadecanoid (ODA) pathway (Fig. 2; [51, 52]). Farmer and Ryan [53] have proposed that a lipase generates $\alpha$-linolenic acid, which is the first precursor in the ODA pathway. $\alpha$-Linolenic acid is then converted by a lipoxygenase, an allene oxide synthase and an allene oxide cyclase into the intermediate 12-oxophytodienoic acid (12-oxo-PDA). This compound is converted into JA through the action of a reductase and three rounds of $\beta$-oxidation. (Me)JA and some of its octadecanoid precursors play key roles as intermediate signals in elicitor-induced secondary metabolite accumulation [54–57]. A correlation between elicitor-induced accumulation of endogenous JA and secondary metabolite accumulation was shown in *Eschscholtzia californica* cells [55] and in rice cells [57]. In parsley cells, phenylpropanoid biosynthetic genes are induced by octadecanoids, and elicitor-induced gene expression is blocked by a lipoxygenase inhibitor [56]. For induction of secondary metabolite accumulation, 12-oxo-PDA may also play a major role. Structural analogues of 12-oxo-PDA that cannot be converted into JA are effective in the induction of alkaloid accumulation [58]. Furthermore, some plant species accumulate more 12-oxo-PDA than JA upon elicitor treatment [59], in support of the hypothesis that 12-oxo-PDA acts as the predominant ODA signal in certain defense responses. Experiments using the *Bryonia dioica* tendril-coiling bioassay for jasmonate also suggest that, in that system, 12-oxo-PDA, rather than JA, has to be considered as the endogenous signal transducer [60].

How elicitors affect JA biosynthesis and how the JA signal is transduced to affect gene expression is largely unknown. Several reports have implicated the activation of a wound-responsive MAPK cascade upstream of JA biosynthesis [61–63]. Downstream of the ODA pathway there are also one or more protein kinases involved in transduction of the JA signal [64]. A protein kinase (cascade) ultimately changes the activity of transcription factors, which regulate the

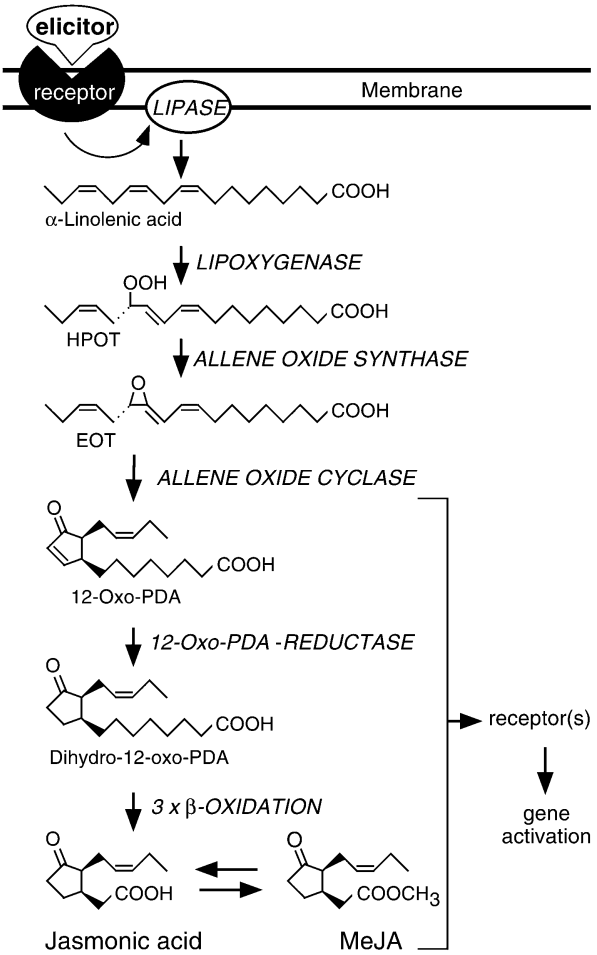


**Fig. 2.** Schematic representation of the jasmonate biosynthetic pathway: *12-oxo-PDA* 12-Oxophytodienoic acid; *HPOT* 13(*S*)-hydroperoxyoctadecatrienoic acid; *EOT* 12,13(*S*)-epoxyoctadecatrienoic acid; *MeJA* methyl jasmonate. Chiral centers have been excluded from the names for clarity. The figure is adapted from Mueller [52]

expression of genes via recognition of specific sequences in the promoter regions. Sequences involved in jasmonate-responsive expression have been identified in the strictosidine synthase gene promoter as discussed below.

## 5 Regulation of the Terpenoid Indole Alkaloid Biosynthetic Pathway

Research on the terpenoid indole alkaloids (TIAs) is mainly primed by the pharmaceutical applications of several of the compounds. The monomeric alkaloids serpentine and ajmalicine are used as a tranquilizer and to reduce hy-

pertension, respectively. The dimeric alkaloids vincristine and vinblastine are potent antitumor drugs. In plants, TIAs are thought to be involved in defence responses. Several reports show that physiological concentrations of TIAs can have antifeeding activity or can delay growth of insect larvae, fungi and microbes [65–67]. Terpenoid indole alkaloids are found in a limited number of plant species belonging to the plant families Apocynaceae, Loganiaceae, Rubiaceae and Nyssaceae. The best progress on molecular characterization of the pathway has been made with *Catharanthus roseus* (L.) G. Don (Madagascar periwinkle), a member of the Apocynaceae family. *C. roseus* cells have the genetic potential to synthesize over 100 terpenoid indole alkaloids.

The initial step in TIA biosynthesis is the condensation of tryptamine with the iridoid glucoside secologanin (Fig. 3). This condensation is performed by the enzyme strictosidine synthase (STR) and results in the synthesis of 3$\alpha$(S)-strictosidine. Strictosidine is the universal precursor of a wide range of different TIAs in various plant species. Additional species-specific enzymes determine the types of TIAs that are formed. In *C. roseus*, strictosidine is deglucosylated by strictosidine $\beta$-D-glucosidase (SGD). Further enzymatic and spontaneous conversions result in the biosynthesis of numerous TIAs. Tryptamine, providing the indole moiety of TIAs, is formed by decarboxylation of tryptophan by the enzyme tryptophan decarboxylase (TDC). The shikimate pathway provides chorismate, which is the precursor of aromatic amino acids. Anthranilate synthase (AS) catalyzes the first step in the conversion of chorismate into tryptophan (Fig. 3). Secologanin, providing the terpenoid part of the TIAs, is synthesized via multiple enzymatic conversions from geraniol. The terpenoid precursors that constitute the backbone of geraniol are produced via the MEP (2-*C*-methyl-D-erythritol 4-phosphate) pathway [68] (formerly called the deoxyxylulose, GAP/pyruvate or Rohmer pathway; [69]). Most steps involved in the conversion of geraniol to secologanin are unknown. Geraniol 10-hydroxylase (G10H), an enzyme of the cytochrome P450 family, catalyzes the first committed step in the formation of secologanin by 10-hydroxylation of geraniol. The enzyme NADPH:cytochrome P450 reductase (CPR) is essential for the activity of G10H and other cytochrome P450 monooxygenases, since it functions in electron transfer to cytochrome P450 proteins. Dimeric alkaloids are formed by peroxidase-catalyzed condensation of vindoline and catharanthine (Fig. 3). Many monomeric TIAs are found in all plant organs, but vindoline and vindoline-derived dimeric alkaloids are only found in chloroplast-containing plant tissues [70]. Vindoline is derived via a number of steps from tabersonine. The first step is catalyzed by the P450 enzyme tabersonine 16-hydroxylase. The two final steps are catalyzed by acetyl CoA:deacetylvindoline 4-*O*-acetyltransferase (DAT) and the 2-oxoglutarate-dependent dioxygenase desacetoxyvindoline-4-hydroxylase (D4H) (Fig. 3).

The biosynthesis of TIAs is highly regulated, and depends on tissue-specific factors as well as environmental signals. Developmental control of TIA biosynthesis and gene expression has recently been reviewed [71]. Here, we will restrict ourselves to stress signals that regulate TIA biosynthesis and gene expression. Inducing effects were observed for fungal elicitors [72], jasmonates [73, 74], auxin starvation [74] and UV-B light [75].

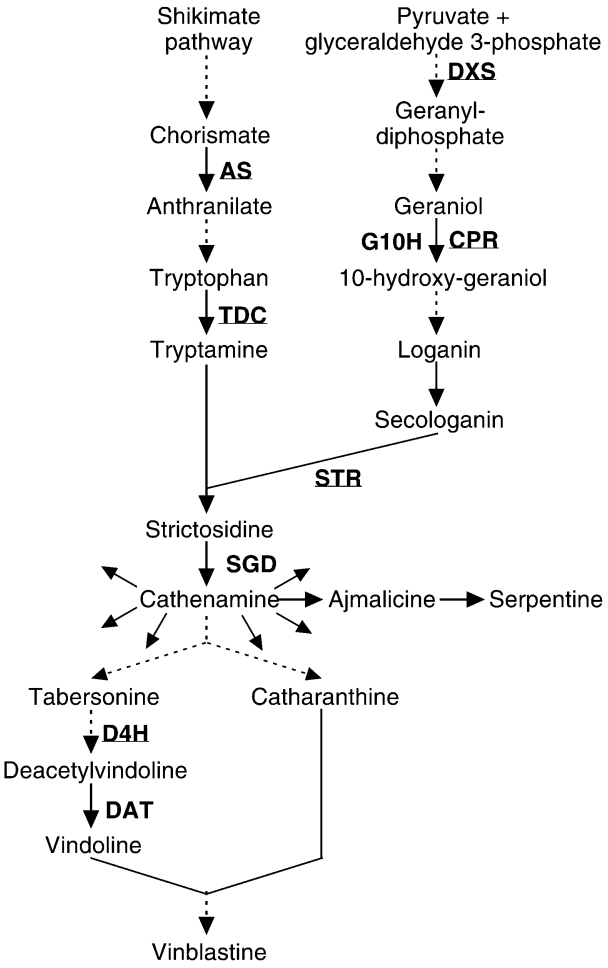


**Fig. 3.** Biosynthesis of TIAs in *C. roseus*. *Solid arrows* indicate single enzymatic conversions, whereas *dashed arrows* indicate multiple enzymatic conversions. *AS* Anthranilate synthase, *DXS* D-1-deoxyxylulose 5-phosphate synthase; *G10H* geraniol 10-hydroxylase; *CPR* cytochrome P450 reductase; *TDC* tryptophan decarboxylase; *STR* strictosidine synthase; *SGD* strictosidine $\beta$-D-glucosidase; *D4H* desacetoxyvindoline 4-hydroxylase; *DAT* acetyl-CoA:4-*O*-deacetylvindoline 4-*O*-acetyl transferase. Genes regulated by ORCA3 are *underlined*. Reprinted with permission from [91]. Copyright (2000) American Association for the Advancement of Science

cDNA clones encoding strictosidine synthase from *Rauwolfia serpentina* [76] and from *C. roseus* [77, 78] and genomic sequences from *R.serpentina* and *R. mannii* [79] and *C. roseus* [80] have been isolated. A *Tdc* cDNA [81] and corresponding genomic clone [82] have been isolated from *C. roseus*. *Str* and *Tdc* mRNA accumulate in suspension-cultured cells after auxin starvation [78, 83], and exposure to fungal elicitors [78, 84] or (methyl) jasmonate [64]. Elicitor-re-

sponsive expression of *Tdc* and *Str* depends on jasmonate as a secondary signal [64]. Elicitor-induced jasmonate biosynthesis requires protein kinase activity [64] and an elevation of cytosolic calcium concentration [85] (Fig. 5). *Tdc* and *Str* in leaves are induced by a UV-B light pulse [75]. As TDC and STR are encoded by single-copy genes in *C. roseus* [78, 82], all these signals act on the same promoter regions. The Chinese tree *Camptotheca acuminata*, on the other hand, contains at least two distinct *Tdc* genes, which are differentially controlled [86]. Accumulation of *Tdc*1 mRNA is developmentally regulated, whereas *Tdc*2 mRNA is induced by fungal elicitor and methyl jasmonate.

In addition, cDNA clones for G10H [87], CPR [88], which is essential for the G10H-catalyzed reaction, and SGD [89] have been isolated. *Cpr* mRNA accumulation is rapidly induced by fungal elicitor [88] and the *Cpr* promoter is elicitor-responsive in transgenic tobacco [90]. All three genes are induced by MeJA in *C. roseus* cell cultures [89, 91].

From the vindoline pathway, cDNA clones for tabersonine 16-hydroxylase [92], DAT [93], and D4H [94] have been isolated. The expression of the corresponding genes is light-regulated in seedlings. In addition, *D4h* and *Dat* have been shown to be induced by MeJA in cell cultures [91].

The observations that *Tdc* and *Str* mRNAs coordinately accumulate in response to fungal elicitors, jasmonates, UV light, and auxin starvation, clearly indicate that the *Tdc* and *Str* genes are controlled by common regulators. Since all the TIA biosynthetic genes tested were induced by MeJA [91], this suggests that a common jasmonate-responsive regulator controls many and possibly all TIA structural genes. Such regulators could prove to be useful to engineer TIA biosynthesis, and the following sections discuss two strategies for their isolation.

## 6 Isolation of Regulatory Genes via Yeast One-Hybrid Screening

The yeast one-hybrid system is a genetic screening method for the isolation of proteins binding to characterized *cis*-acting promoter elements (Fig. 4). In this approach, a yeast strain is constructed carrying a reporter gene driven by an artificial promoter, with a bait composed of multimers of the promoter element of interest fused to a yeast TATA box region. Upon transformation of a cDNA library, cloned as a translational fusion with the heterologous GAL4 activation domain, yeast cells are selected for reporter gene activation. Since the resulting clones encode fusion proteins consisting of the cDNA-encoded protein and the GAL4 activation domain, cDNAs are solely selected for binding of the corresponding proteins to the promoter element of interest. This system has been used successfully to clone several transacting factors from mammalian systems and plants [48, 95].

The *Str* promoter has been extensively studied to identify elicitor- and jasmonate-responsive sequences [48, 80, 95]. Two regions were identified that dictated elicitor- and jasmonate-responsive reporter gene activation, the so-called BA region (Fig. 5; [48]), and a region close to the TATA box called jasmonate- and elicitor-responsive element (JERE; [95]). An *Str* promoter derivative with a deletion of the BA region is still active and responsive to stress signals, but at a

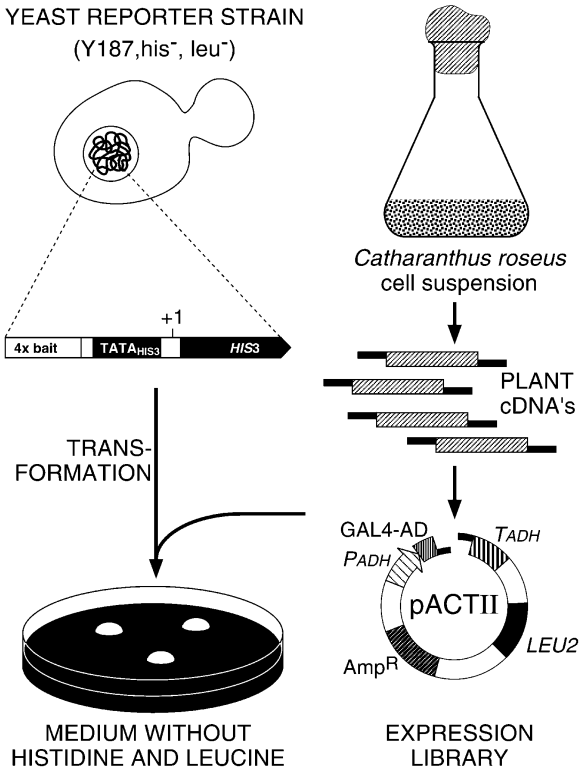


**Fig. 4.** Schematic representation of yeast one-hybrid transcription factor screening. *C. roseus* cDNAs were cloned in a fusion with the GAL4-activation domain (GAL4-AD) in yeast/*E.coli* shuttle vector pACTII. Yeast reporter strains carrying a tetramer of the *cis*-acting elements of interest (indicated in the figure with 4xbait) were transformed with the cDNA library and selected for transformation and reporter gene activation on medium lacking leucine and histidine, respectively

lower level. Mutation or removal of the JERE results in an inactive and unresponsive *Str* promoter derivative [95].

Yeast one-hybrid screening with the JERE as a bait identified two transcription factors of the AP2/ERF-domain family that were called ORCA1 and ORCA2 (*O*ctadecanoid-*R*esponsive *Catharanthus A*P2; [95]). The AP2/ERF transcription factors are unique to plants, and are characterized by the AP2/ERF DNA-binding domain [96, 97]. *Orca2* gene expression was induced by MeJA and elicitor. Furthermore, the ORCA2 protein showed sequence-specific binding to and transactivated *Str* gene expression via the JERE [95]. In contrast to *Orca2*, *Orca1* gene expression was not induced by MeJA and elicitor, indicating that ORCA1 is not involved in regulation of JA- and elicitor-induced *Str* expression.

Use of the BA region in a yeast one-hybrid screen resulted in the isolation of a periwinkle homologue of the MYB-like factor BPF-1 from parsley [48]. Like its parsley counterpart [47], transcription of periwinkle *CrBPF-1* is induced by

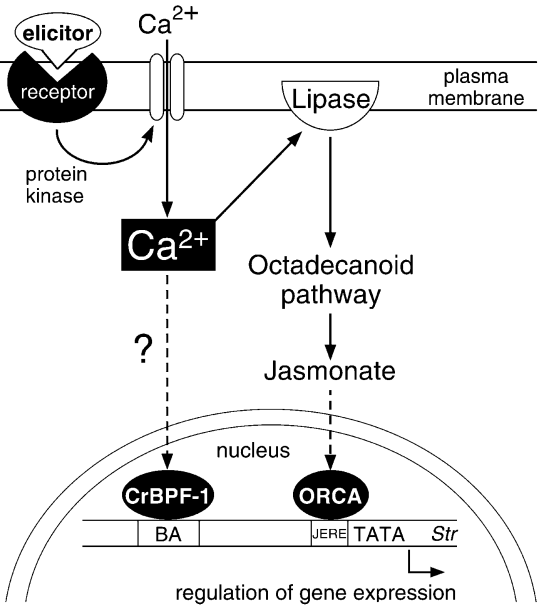


**Fig. 5.** Model for elicitor signal transduction leading to *Str* expression. The model shows the positions of CrBPF-1 in a JA-independent, and ORCAs in a JA-dependent, elicitor signal transduction pathway. Protein phosphorylation and calcium influx are required for elicitor-induced jasmonate biosynthesis, as well as for the induction of *CrBPF-1*, *ORCA2*, and *ORCA3*. ORCA activation additionally depends on jasmonate biosynthesis. The positions of the TATA box, the BA region, and the jasmonate- and elicitor-responsive element (JERE) within the *Str* promoter are indicated

fungal elicitor. *CrBPF1* elicitation depends on protein kinase activity and a rise in cytosolic calcium concentration, but is independent of jasmonate biosynthesis (Fig. 5).

Figure 5 shows a model that summarizes activation of *Str* gene expression by elicitor. Upon perception of the elicitor, a protein phosphorylation step is required to induce a calcium influx. This transient increase in cytosolic [$Ca^{2+}$] is necessary for activation of the ODA pathway [85]. Jasmonate induces *Orca2* gene expression [95]. ORCA2 then activates *Str* expression via interaction with the JERE. In addition, calcium influx is required for JA-independent induction of *CrBPF-1* mRNA accumulation, putatively involved in regulation of *Str* via interaction with the jasmonate- and elicitor-responsive BA region. Deletion of the BA fragment from the *Str* promoter did not abolish elicitor and jasmonate responsiveness of this promoter, whereas deletion or mutation of the JERE within the –339 promoter context resulted in a promoter derivative that is inactive and lacks elicitor and JA responsiveness [95]. Therefore, CrBPF-1 cannot be sufficient for elicitor-induced expression of *Str*. It can be speculated that the JERE of the *Str* promoter forms a switch for elicitor and jasmonate responsiveness, and that other *cis*-elements with their cognate binding factors (such as BA/CrBPF-1) are important for enhancing the elicitor and/or jasmonate response.

## 7
## Isolation of Regulatory Genes via T-DNA Activation Tagging

One of the most direct ways to find regulators of metabolism is the generation and analysis of genetic mutants. Mutations resulting from chemical mutagenesis or tagging with transposons or T-DNAs usually cause loss of function, and are therefore recessive. Consequently, the mutant phenotype can only be visualized following selfing of the mutated plants. This demands a substantial amount of effort, and is not possible for all plant species. Another drawback of classical mutagenesis is that mutation of functionally redundant genes does not result in phenotypically altered plants. In contrast, mutants generated by T-DNA activation tagging are dominant, allowing direct selection of the desired phenotype in the primary transformants. Furthermore, a phenotype can result from T-DNA activation tagging of a functionally redundant gene, allowing its cloning and functional analysis. The T-DNA activation tagging approach consists of transformation of plants or plant cells with a T-DNA, which carries promoter elements reading towards one of its borders. Upon random integration in the plant genome, flanking plant sequences can be transcribed, which can result in a dominant mutation (Fig. 6). T-DNA activation tagging has been used to isolate genes involved in the timing of flowering [98, 99], or in responses to abscisic acid [100] or cytokinin [101].

This strategy was successfully applied to isolate a regulator of TIA biosynthesis. *C. roseus* cells transformed with a T-DNA tag were selected on a toxic level of 4-methyltryptophan. This amino acid analogue can be converted by the TDC enzyme into its nontoxic derivative 4-methyltryptamine [102]. This selection strategy therefore will select T-DNA tagged cells with elevated levels of TDC enzyme activity (Fig. 6). In addition, cells can survive 4-mT selection by producing more tryptophan, by reduced 4-mT uptake, and many other possible mechanisms. To select for regulators of *Tdc* gene expression, 4-mT resistant cell

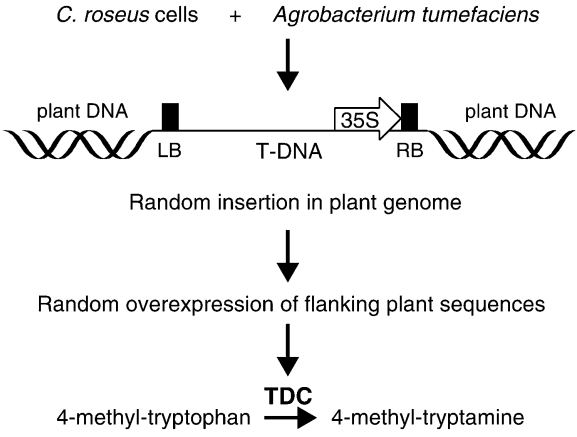


**Fig. 6.** Schematic representation of the T-DNA tagging approach to identify regulators of TIA biosynthetic genes in *C. roseus*. For explanation, see the text

lines were screened for high *Tdc* mRNA levels via Northern blot hybridization. To select for cell lines in which the T-DNA tag activated a master regulator of TIA biosynthetic gene expression, cell lines with high *Tdc* mRNA levels were subsequently screened for high *Str* mRNA levels (Fig. 7). In total 400,000 to 500,000 independent cells, stably transformed with a T-DNA construct carrying CaMV 35S enhancer sequences reading towards the right border sequence, were selected for 4-mT resistance. The haploid genome size of *C. roseus* is $8 \times 10^8$ bp according to a recent estimation (Frank van Iren, personal communication). This is 2.5-fold smaller than a previous estimation [103]. Thus, by generation of 400,000 to 500,000 independent transformants with on average two T-DNA copies per genome [104], the probability of finding a T-DNA insert in a given 2 kb DNA region is around 90% using the formula $P = 1 - (1 - [x/800.000])^n$ (where $P$ = probability; $x$ = length of DNA region in kb; $n$ = number of T-DNA insertions in population; and T-DNA integration is assumed to be random). Since T-DNA

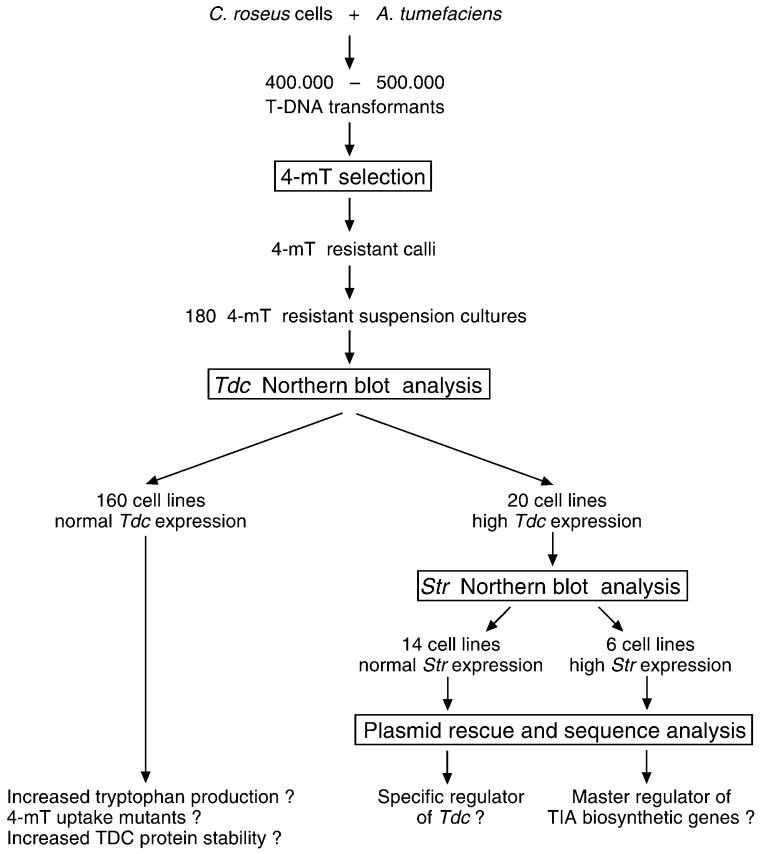


**Fig. 7.** Schematic representation of the selection procedure and the numbers of transformants and mutants that were generated. Possible mechanisms for passing the specific selection steps are indicated at the bottom of the figure

preferentially inserts into transcribed regions of the plant genome, T-DNA density near or into genes may even be higher. The CaMV 35S promoter has been shown to activate gene expression over a distance of at least 3.6 kb in an activation tagging approach [99]. Based on these calculations, it can be concluded that T-DNA activation tagging of the *C. roseus* genome by construct Tag-2B4A1 was close to saturation. After several weeks of selection, 4-mT resistant calli were obtained. To generate sufficient biomass for subsequent analysis, suspension cultures were generated from 180 calli. Further screening of 4-mT resistant cell lines for high *Tdc* expression by Northern blot analysis identified 20 lines with increased *Tdc* mRNA accumulation. Thus, only approximately 10% of the 4-mT resistant lines showed the desired phenotype. The other 90% of the lines were 4-mT resistant via a mechanism different from increased *Tdc* expression; for example, by increased tryptophan production, by impaired 4-mT uptake, or by increased TDC protein stability.

The 4-mT resistant cell lines were further checked for increased expression of *Str*, a TIA biosynthetic gene that was not used as selectable marker in the initial selection. Only six lines showed, in addition to increased *Tdc* expression, enhanced *Str* mRNA accumulation, suggesting that a component of a shared signal transduction pathway leading to regulation of several, and possibly all, TIA biosynthetic genes was tagged in these lines. Thus, the frequency of tagging central signaling components was low. Production of secondary metabolites and active cell proliferation appear to be mutually exclusive processes. Since the T-DNA activation tagging approach selected for actively growing cells, it can be speculated that there was a negative selection pressure against cells with increased expression of the complete TIA biosynthetic pathway and consequent production of secondary metabolites. Alternatively, the number of target genes that act as master regulators for multiple genes operating in the TIA biosynthetic pathway may be limited.

For one of the six lines with increased expression of *Tdc* and *Str* (line 46), the isolation of the T-DNA-flanking plant DNA by plasmid rescue was successful. Subsequent characterization of the rescued DNA showed that the T-DNA tag activated a gene encoding another AP2/ERF-domain transcription factor, which was called ORCA3 [91]. ORCA3 shows a high similarity to ORCA2 in the AP2/ERF DNA-binding domain, but no amino acid identity elsewhere was observed. The expression of the *Orca3* gene was induced by MeJA with similar kinetics as *Orca2* expression [105]. The ORCA3 protein bound the *Str*, *Tdc* and *Cpr* promoters, and increased their transcriptional activity in a transient assay [91]. The activation of the *Str* promoter depended on a specific interaction of ORCA3 with the JERE [105]. The effect of ORCA3 on metabolism is described in the next section.

## 8 Modification of TIA Metabolism Using the Transcription Factor ORCA3

In line 46, overexpressing ORCA3 due to the inserted T-DNA activation tag, expression of TIA biosynthetic genes *Tdc*, *Str*, *Sgd, Cpr* and *D4h* was increased (schematically depicted in Fig. 3). TIA biosynthetic genes *G10h* and *Dat* were

not induced, suggesting that these genes are not controlled by ORCA3. Genes encoding the $\alpha$ subunit of AS (AS$\alpha$) and DXS, enzymes involved in primary metabolism leading to TIA precursor synthesis, were induced by *Orca3* overexpression, whereas two other primary metabolic genes not involved in production of TIA precursors (*Ggpps* and *Ics)* were not regulated by ORCA3. Gene expression patterns observed in cell lines that were independently transformed by particle bombardment with the rescued *Orca3* gene under control of CaMV 35S promoter elements were identical to that of line 46. These results indicate that ORCA3 is a regulator of primary as well as secondary metabolite biosynthetic genes involved in TIA biosynthesis. Although previous studies report differential regulation of genes involved in early (i.e. *Tdc* and *Str*) and late (i.e. *D4h*) steps in vindoline biosynthesis in periwinkle plants [71, 106], genes of both classes are controlled by *Orca3* in suspension-cultured cells.

Plant cells must regulate their primary metabolic pathways to accommodate the biosynthesis of secondary metabolites. Previously, a strong correlation between induction of tryptophan biosynthetic gene expression and indolic phytoalexin accumulation was observed in *Arabidopsis* plants [107], indicating coordinate regulation of these processes. In *C. roseus* genes involved in both primary and secondary metabolic pathways can be regulated by a single AP2/ERF-domain transcription factor, ORCA3. Thus, ORCA3 acts as a central regulator to direct metabolic fluxes into the TIA biosynthetic pathway by regulation of expression of genes involved in primary as well as secondary metabolism.

All primary and secondary metabolite biosynthetic genes that were tested, together with *Orca3* itself, were induced by MeJA [91]. However, not all MeJA-induced genes were regulated by *Orca3*. For example, expression of *G10h* was strongly induced by MeJA, whereas *G10h* expression was not detected in *Orca3* overexpressing cell lines, suggesting that additional jasmonate-responsive transcription factors are involved in regulation of this gene. A possible candidate for this regulation is ORCA2.

Cell cultures overexpressing *Orca3* accumulated significantly increased amounts of tryptamine, providing the indole moiety of TIAs. Since no TIAs were detected in these cultures, it was concluded that the terpenoid part was limiting for TIA production, and that this limitation could not be overcome by *Orca3* overexpression. A possible bottleneck was *G10h*, since *G10h* expression was not observed in the *Orca3* overexpressing cell cultures. When the terpenoid precursor loganin was added to the cell cultures, *Orca3* overexpression caused an increase in TIA production [91]. A significant increase in TIA biosynthesis has never been conclusively shown upon overexpression of *Tdc*, *Str* or both genes, even after feeding with terpenoid precursors [108, 109]. This clearly demonstrates the benefit of using central regulators for metabolic engineering of plant cells.

## 9 Conclusions

The examples discussed here show that for two secondary metabolite pathways, metabolic engineering using transcription factors was successful. Modification of the phenylpropanoid/flavonoid pathway made use of the tissue-specific MYB

and bHLH transcription factors, whereas for modulation of the TIA biosynthetic pathway, a jasmonate-responsive AP2/ERF-domain transcription factor was used.

Overexpression experiments with the MYB and bHLH protein-encoding genes have demonstrated that flavonoid biosynthesis can be engineered using transcription factors. The MYB and bHLH factors are active over species borders and faithfully recognize their orthologous target genes in heterologous species. In this respect the MYB and bHLH proteins behave as master regulators of the flavonoid pathway, although maybe not in all plant species to a similar extent. Ectopic expression of *C1* and *R* constitutes a viable strategy for engineering anthocyanin production in plant cell cultures [50].

Overexpression of the jasmonate-responsive AP2/ERF-domain transcription factor ORCA3 resulted in elevated levels of tryptophan and tryptamine, and, upon feeding of a terpenoid precursor, elevated levels of certain TIAs [91].

AP2/ERF-domain transcription factors occupy central positions in regulation of plant stress responses [96]. ERF1, for example, is an ethylene-responsive AP2/ERF-domain protein (Fig. 8). Its overexpression resulted in induction of several ethylene-responsive defense genes in *Arabidopsis thaliana* [110]. In tomato, Pto is a receptor kinase that is involved in the recognition of *Pseudomonas* avirulence gene product AvrPto [111]. Yeast two-hybrid screening identified three Pto-interacting (Pti4, 5 and 6) tomato proteins, each of which contained an AP2/ERF domain [112]. Pti4, 5 and 6 are thought to be involved in elicitor-induced activation of defense genes in tomato (Fig. 8). Another family of AP2/ERF-domain proteins, represented by DREB2A and DREB2B, is involved in drought-responsive gene expression [113], whereas the CBF/DREB1 family includes central regulators of cold-regulated gene expression in *Arabidopsis* [9, 113] (Fig. 8). Upon overexpression of the AP2/ERF-domain protein CBF1, the

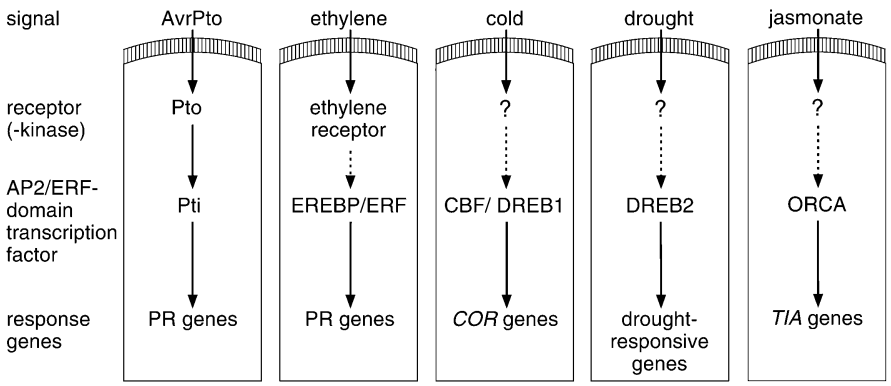


**Fig. 8.** Models showing the involvement of AP2/ERF-domain transcription factors in regulation of stress and defense gene expression in plants. The stress signal is perceived by interaction with a receptor (-kinase). Induction of de novo synthesis and/or modulation of the preexisting AP2/ERF-domain transcription factor activate gene expression. Unidentified signal transduction components are indicated with a *question mark*. *Dashed arrows* indicate the possible involvement of multiple signalling steps

expression of a set of cold-induced genes was enhanced, thereby increasing freezing tolerance [9]. In *C. roseus* the expression of TIA biosynthetic genes is coordinately induced by jasmonates [64, 89, 91]. A considerable number of these genes are controlled by ORCA3 [91]. Therefore, the spectrum of defense genes regulated by AP2/ERF-domain transcription factors includes genes involved in jasmonate-responsive secondary metabolism (Fig. 8).

Since several plant secondary metabolic pathways are regulated by jasmonate, it is tempting to speculate that these pathways in other plant species are controlled by ORCA-like AP2/ERF-domain transcription factors. Therefore, identification and isolation of *Orca* homologues might offer opportunities for genetic engineering of JA-responsive metabolism in plants in general. Overexpression of maize MYB and bHLH regulatory proteins in heterologous plant species resulted in increased production of secondary metabolites [21], demonstrating the possibilities for interspecies exchange of regulatory proteins. Expression of *C. roseus* ORCA proteins in other plant species might result in induction of jasmonate-responsive metabolic pathways, and, subsequently, in increased metabolite production.

Although ORCA3 regulates multiple genes in primary and secondary metabolism, not all genes in TIA metabolism are controlled by ORCA3. Possibly, other transcription factors are involved in controlling these genes. A good candidate is ORCA2, another jasmonate-responsive AP2/ERF-domain transcription factor from *C. roseus*. Preliminary results indicate that ORCA2 and ORCA3 have overlapping, but distinct, sets of target genes. This indicates that these ORCA proteins have different functions, and that both are required for the full spectrum of jasmonate-induced metabolic changes. Future experiments using a combination of ORCA2 and ORCA3 should establish to what extent they regulate the TIA biosynthetic pathway, and whether additional transcription factors may be involved.

In using central regulators to engineer secondary metabolism, the use of inducible promoters deserves attention, because constitutive expression of a pleiotropic regulator, or a high constitutive level of certain secondary metabolites, may not be compatible with cell viability. In addition, transcriptional regulators may be engineered to make them dominant and independent of the normal control of their activity. This may be especially important for central regulators that are activated by stress signals. The two approaches may be combined by constructing hybrid transcription factors, that consist of the DNA-binding domain to direct them to their target genes, a strong transactivation domain, and an inducible domain that silences the activation domain in the uninduced state. Such an approach was used to produce anthocyanins in the *Arabidopsis ttg* mutant that lacks anthocyanins. Expression of a fusion between the DNA-binding domain of the maize bHLH protein *R* and the steroid-binding domain of the rat glucocorticoid receptor restored anthocyanin production in a steroid hormone-dependent manner [114]. In addition, inducible expression of transcription factors provides a valuable tool for the identification of their target genes [115].

## References


1. Verpoorte R, van der Heijden R, Memelink J (1998) Plant biotechnology and the production of alkaloids: prospects of metabolic engineering. In: Cordell GA (ed) The alkaloids, vol 50. Academic Press, San Diego, p 453
2. Verpoorte R, van der Heijden R, ten Hoopen HJG, Memelink J (1999) Biotechnol Lett 21:467
3. Verpoorte R, van der Heijden R, Memelink J (2000) General strategies. In: Verpoorte R, Alfermann AW (eds) Metabolic engineering of plant secondary metabolism. Kluwer, Dordrecht, p 31
4. Nikolov DB, Burley SK (1997) Proc Natl Acad Sci USA 94:15
5. Kaiser K, Meisterernst M (1996) Trends Biochem Sci 21:342
6. Molkentin JD, Olson EN (1996) Proc Natl Acad Sci USA 93:9366
7. Lawrence PA, Morata G (1994) Cell 78:181
8. Graba Y, Aragnol D, Pradel J (1997) BioEssays 19:379
9. Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998) Science 280:104
10. Holton TA, Cornish EC (1995) Plant Cell 7:1071
11. Roth BA, Goff SA, Klein TM, Fromm ME (1991) Plant Cell 3:317
12. Moyano E, Martinez-Garcia JF, Martin C (1996) Plant Cell 8:1519
13. Sainz MB, Grotewold E, Chandler VL (1997) Plant Cell 9:611
14. Martin C, Paz-Ares J (1997) Trends Genet 13:67–73
15. Jin H, Martin C (1999) Plant Mol Biol 41:577
16. de Pater S, Pham K, Memelink J, Kijne J (1997) Plant Mol Biol 34:169
17. Martínez-García JF, Huq E, Quail PH (2000) Science 288:859
18. Menkens AE, Schindler U, Cashmore AR (1995) Trends Biochem Sci 20:506
19. Goff SA, Cone KC, Chandler VL (1992) Genes Dev 6:864
20. Mol J, Grotewold E, Koes R (1998) Trends Plant Sci 3:212
21. Lloyd AM, Walbot V, Davis RW (1992) Science 258:1773
22. Bradley JM, Davies KM, Deroles SC, Bloor SJ, Lewis DH (1998) Plant J 13:381
23. Ludwig SR, Wessler SR (1990) Cell 62:849
24. de Vetten N, Quattrocchio F, Mol J, Koes R (1997) 11:1422
25. Walker AR, Davison PA, Bolognesi-Winfield AC, James CM, Srinivasan N, Blundell TL, Esch JJ, Marks MD, Gray JC (1999) Plant Cell 11:1337
26. McCarthy DR, Carson CB, Stinard PS, Robertson DS (1989) Plant Cell 1:523
27. Hattori T, Vasil V, Rosenkrans L, Hannah LC, McCarty DR, Vasil IK (1992) Genes Dev 6:609
28. Suzuki M, Kao CY, McCarty DR (1997) Plant Cell 9:799
29. Blackwood EM, Eisenman RN (1991) Science 251:1211
30. Gu W, Bhatia K, Magrath IT, Dang CV, Dalla-Favera R (1994) Science 264:251
31. Hunter T, Karin M (1992) Cell 70:375
32. Lüscher B, Cristenson E, Litchfield DW, Krebs EG, Eisenman RN (1990) Nature 344:517
33. Myrset AH, Bostad A, Jamin N, Lirsac PN, Toma F, Gabrielsen OS (1993) EMBO J 12:4625
34. Oelgeschläger M, Janknecht R, Krieg J, Schreek S, Luescher B (1996) EMBO J 15:2771
35. Dai P, Akimaru H, Tanaka Y, Hou DX, Yasukawa T, Kanei-Ishii C, Takahashi T, Ishii S (1996) Genes Dev 10:528
36. Dixon RA, Paiva NL (1995) Plant Cell 7:1085
37. Schulze-Lefert P, Dangl JL, Becker-André M, Hahlbrock K, Schulz W (1989) EMBO J 8:651
38. Dron M, Clouse SD, Dixon RA, Lawton MA, Lamb CJ (1988) Proc Natl Acad Sci USA 85:6738
39. Lois R, Dietrich A, Hahlbrock K, Schulz W (1989) EMBO J 8:1641
40. Becker-André M, Schulze-Lefert P, Hahlbrock K (1991) J Biol Chem 266:8551
41. Weisshaar B, Armstrong GA, Block A, da Costa e Silva O, Hahlbrock K (1991) EMBO J 10:1777

42. Hartmann U, Valentine WJ, Christie JM, Hays J, Jenkins GI, Weisshaar B (1998) Plant Mol Biol 36:741
43. Ang L-H, Chattopadhyay S, Wei N, Oyama K, Batschauer A, Deng XW (1998) Mol Cell 1:213
44. Sablowski RWM, Moyano E, Culianez-Marcia FA, Schuch W, Martin C, Bevan M (1994) EMBO J 13:128
45. Feldbrügge M, Sprenger M, Hahlbrock K, Weisshaar B (1997) Plant J 11:1079
46. Dröge-Laser W, Kaiser A, Lindsay WP, Halkier BA, Loake GJ, Doerner P, Dixon RA, Lamb C (1997) EMBO J 16:726
47. da Costa e Silva O, Klein L, Schmelzer E, Trezzini GF, Hahlbrock K (1993) Plant J 4:125
48. van der Fits L, Zhang H, Menke FLH, Deneka M, Memelink J (2000) Plant Mol Biol 44:675
49. Mooney M, Desnos T, Harrison K, Jones J, Carpenter R, Coen E (1995) Plant J 7:333
50. Grotewold E, Chamberlin M, Snook M, Siame B, Butler L, Swenson J, Maddock S, St. Clair G, Bowen B (1998) Plant Cell 10:721
51. Vick BA, Zimmerman DC (1984) Plant Physiol 75:458
52. Mueller MJ (1997) Physiol Plant 100:653
53. Farmer EE, Ryan CA (1992) Plant Cell 4:129
54. Gundlach H, Müller MJ, Kutchan TM, Zenk MH (1992) Proc Natl Acad Sci USA 89:2389
55. Mueller MJ, Brodschelm W, Spannagl E, Zenk MH (1993) Proc Natl Acad Sci USA 90:7490
56. Ellard-Ivey M, Douglas CJ (1996) Plant Physiol 112:183
57. Nojiri H, Sugimori M, Yamane H, Nishimura Y, Yamada A, Shibuya N, Kodama O, Murofushi N, Omori T (1996) Plant Physiol 110:387
58. Blechert S, Brodschelm W, Hölder W, Kammerer L, Kutchan TM, Mueller MJ, Xia Z-Q, Zenk MH (1995) Proc Natl Acad Sci USA 92:4099
59. Parchmann S, Gundlach H, Mueller MJ (1997) Plant Physiol 115:1057
60. Blechert S, Bockelmann C, Füßlein M, v Schrader T, Stelmach B, Niesel U, Weiler EW (1999) Planta 207:470
61. Seo S, Okamoto M, Seto H, Ishizuka K, Sano H, Ohashi Y (1995) Science 270:1988
62. Seo S, Sano H, Ohashi Y (1999) Plant Cell 11:289
63. Stratmann JW, Ryan CA (1997) Proc Natl Acad Sci USA 94:11085
64. Menke FLH, Parchmann S, Mueller MJ, Kijne JW, Memelink J (1999) Plant Physiol 119:1289
65. Aerts RJ, Snoeijer W, Aerts-Teerlink O, van der Meijden E, Verpoorte R (1991) Phytochemistry 30:3571
66. Aerts RJ, Stoker A, Beishuizen M, Jaarsma I, van de Heuvel M, van der Meijden E, Verpoorte R (1992) J Chem Ecol 18:1955
67. Luijendijk TJC (1995) PhD thesis, Leiden University, The Netherlands
68. Contin A, van der Heijden R, Lefeber AWM, Verpoorte R (1998) FEBS Lett 434:413
69. Rohmer M, Knani M, Simonin P, Sutter B, Sahm H (1993) Biochem J 295:517
70. Meijer AH, Verpoorte R, Hoge JHC (1993) J Plant Res 3:145
71. De Luca V, St-Pierre B (2000) Trends Plant Sci 5:168
72. Moreno PRH, van der Heijden R, Verpoorte R (1995) Plant Cell Tiss Org Cult 42:1
73. Aerts RJ, Gisi D, De Carolis E, De Luca V, Baumann TW (1994) Plant J 5:635
74. Gantet P, Imbault N, Thiersault M, Doireau P (1998) Plant Cell Physiol 39:220
75. Ouwerkerk PBF, Hallard D, Verpoorte R, Memelink J (1999) Plant Mol Biol 41:491
76. Kutchan TM, Hampp N, Lottspeich F, Beyreuther K, Zenk MH (1988) FEBS Lett 237:40
77. McKnight TD, Roessner CA, Devagupta R, Scott AI, Nessler CL (1990) Nucl Acids Res 18:4939
78. Pasquali G, Goddijn OJM, de Waal A, Verpoorte R, Schilperoort RA, Hoge JHC, Memelink J (1992) Plant Mol Biol 18:1121
79. Bracher D, Kutchan TM (1992) Arch Bioch Biophys 294:717
80. Pasquali G, Erven ASW, Ouwerkerk PBF, Menke FLH, Memelink J (1999) Plant Mol Biol 39:1299

81. De Luca V, Marineau C, Brisson N (1989) Proc Natl Acad Sci USA 86:2582
82. Goddijn OJM, Lohman FP, de Kam RJ, Schilperoort RA, Hoge JHC (1994) Mol Gen Genet 242:217
83. Goddijn OJM, de Kam RJ, Zanetti A, Schilperoort RA, Hoge JHC (1992) Plant Mol Biol 18:1113
84. Roewer IA, Cloutier C, Nessler CL, De Luca V (1992) Plant Cell Rep 11:86
85. Menke FLH (1999) PhD thesis, Leiden University, The Netherlands
86. López-Meyer M, Nessler CL (1997) Plant J 11:1167
87. Collu G (1999) PhD thesis, Leiden University, The Netherlands
88. Meijer AH, Lopes Cardoso MI, Voskuilen JT, de Waal A, Verpoorte R, Hoge JHC (1993) Plant J 4:47
89. Geerlings A, Martinez-Lozano Ibanez M, Memelink J, van der Heijden R, Verpoorte R (2000) J Biol Chem 275:3051
90. Lopes Cardoso MI, Meijer AH, Rueb S, Queiroz Machado J, Memelink J, Hoge JHC (1997) Mol Gen Genet 256:674
91. van der Fits L, Memelink J (2000) Science 289:295
92. Schröder G, Unterbusch E, Kaltenbach M, Schmidt J, Strack D, De Luca V, Schröder J (1999) FEBS Lett 458:97
93. St-Pierre B, Laflamme P, Alarco A-M, De Luca V (1998) Plant J 14:703
94. Vazquez-Flota F, De Carolis E, Alarco A-M, De Luca V (1997) Plant Mol Biol 34:935
95. Menke FLH, Champion A, Kijne JW, Memelink J (1999) EMBO J 18:4455
96. Riechmann JL, Meyerowitz EM (1998) Biol Chem 379:633
97. Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M (2000) Plant Cell 12:393
98. Kardailsky I, Shukla VK, Ahn JH, Dagenais N, Christensen SK, Nguyen JT, Chory J, Harrison MJ, Weigel D (1999) Science 286:1962
99. Weigel D, Ahn JI, Blázquez MA, Borevitz JO, Christensen SK, Fankhauser C, Ferrándiz C, Kardailsky I, Malancharuvil EJ, Neff MM, Nguyen JT, Sato S, Wang Z-Y, Xia Y, Dixon RA, Harrison MJ, Lamb CJ, Yanofsky MF, Chory J (2000) Plant Physiol 122:1003
100. Furini A, Koncz C, Salamini F, Bartels D (1997) EMBO J 16:3599
101. Kakimoto T (1996) Science 274:982
102. Sasse F, Buchholz M, Berlin J (1983) Z Naturforsch 38c:910
103. Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1993) Science 220:1049
104. van der Fits L, Deakin EA, Hoge JHC, Memelink J (2000) Plant Mol Biol 43:495
105. van der Fits L, Memelink J (2001) Plant J 25:43
106. St-Pierre B, Vazquez-Flota FA, De Luca V (1999) Plant Cell 11:887
107. Zhao J, Last RL (1996) Plant Cell 8:2235
108. Canel C, Lopes Cardoso MI, Whitmer S, van der Fits L, Pasquali G, van der Heijden R, Hoge JHC, Verpoorte R (1998) Planta 205:414
109. Whitmer S (1999) PhD thesis, Leiden University, The Netherlands
110. Solano R, Stepanova A, Chao Q, Ecker JR (1998) Genes Dev 12:3703
111. Martin GB, Brommonschenkel S, Chunwongse J, Frary A, Ganal MW, Spivey R, Wu T, Earle ED, Tanksley SD (1993) Science 262:1432
112. Zhou J, Tang X, Martin GB (1997) EMBO J 16:3207
113. Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Plant Cell 10, 1391
114. Lloyd AM, Schena M, Walbot V, Davis RW (1994) Science 266:436
115. Bruce W, Folkerts O, Garnaat C, Crasta O, Roth B, Bowen B (2000) Plant Cell 12:65

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# The Production of Foreign Proteins from Genetically Modified Plant Cells

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While traditionally used to produce natural products, plant suspension cultures can also be utilized for the production of foreign proteins. Production of these high-value products in plant cells is an economically viable alternative to other systems, particularly in cases where the protein must be biologically active. There are several advantages to using plant cells for the large-scale production of secreted proteins. Plant cell media are composed of simple sugars and salts and are therefore less expensive and complex than mammalian media. Consequently, purification of secreted protein is simpler and more economical. Additionally, plant cell derived proteins are likely to be safer than those derived from other systems, since plant cell pathogens are not harmful to humans. In this chapter, we will review foreign protein production from plant cells. To begin, we will discuss the behavior of plant cell cultures, products produced by plant cells, protein secretion and its relationship to purification, and the performance of plant cells as compared to whole plants and other alternative hosts. After a brief discussion of gene transfer techniques, we will present strategies to overcome the limitations of protein production, including protein stabilization, novel production schemes, modeling, and scale-up considerations. To conclude, we will discuss implications for future development of this technology.

**Keywords.** Plant cell, Foreign protein, Transgenic, Secretion, Increased production



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# 1 Introduction

## 1.1 Overview

This chapter addresses the emerging use of plant cell culture as a vehicle for foreign protein production. This technology is a combination of the techniques used to produce natural products using cultured plant cells and those used to produce recombinant proteins in whole plants or other hosts. Plant cell culture is a long-established technique for producing flavors, colors, and other important natural products. For example, plant cell culture has been used industrially to produce shikonin, a red pigment with anti-inflammatory properties [1]. However, the lower growth rates and the lower productivity of plant cells limit the applicability of using cultured plant cells to produce such products. In gen-

eral, economic factors limit the use of large-scale plant cell culture to the production of rare, high-value substances. Based on this need, therapeutic protein production is a good candidate for the utilization of plant cell cultures.

The expression of foreign proteins in plant cells is conceptually similar to protein expression in other host systems, but differs in the details. These differences are the source for both the "virtues" and "vices" of the plant cell production system. Tobacco and other plant cells may be genetically transformed by well-defined means and are highly stable. Since they are derived from higher organisms, plant cells have the capacity to carry out any desired post-transcriptional or post-translational modifications on the transcribed RNA or protein of interest, including intron removal, glycosylation, disulfide bond formation, and protein folding events. As high eukaryotic cells they are also capable of recognizing and cleaving protein leader sequences, a trait that facilitates protein-targeting strategies such as secretion or localization within organelles. Unlike many other higher cell types, plant cells grow easily in suspensions and can be grown to very high biomass densities. However, plant cells exhibit slow growth rates and more complex control of gene expression pathways. These (and perhaps other unknown) traits lead to low protein expression levels, typically in the range of 0.1 – 1 mg/l, for plant cell based systems.

The history of work in plant cell protein expression can be divided into three phases. The first phase of research focused on confirming the feasibility of this technology: developing usable constructs, DNA-transfer techniques, and expressing simple reporter proteins. The second phase focused on establishing the usefulness of these methods: improving early techniques, establishing standard protocols, and expressing proteins with measurable biological activity and economic value. The third and most critical phase of the research will be demonstrating economic feasibility: increasing the expression level of useful protein products, carrying out complete purification of plant-produced proteins, conducting successful *in vitro* and clinical studies, and scaling up these procedures for cost comparison with other available hosts. To meet these challenges it will be crucial to combine current knowledge with innovative strategies.

## 1.2 Significance

Plant cell culture is a safe and useful production scheme and an important alternative to other protein production systems. Plant cells are uniquely suited for the safe production of protein-based drugs because, unlike microbial and mammalian cells, they are not a potential source of dangerous pathogens. Because of their high economic value, recombinant proteins are among the best candidate products for commercial production in plant cell culture. The accumulating body of research over the past two decades suggests that plant cell culture is a workable system for producing a variety of gene products. Almost without exception, plant-produced proteins are correctly folded and biologically active. Thus, at its present state, plant cell culture could be used as an in-house protein production source for protein characterization studies or to produce sufficient material for early clinical trials. The study of protein production in plant cells is

also justified as a means for understanding the behavior of protein production mechanisms in plant cells and for identifying plant-derived therapeutics – including proteins. In fact, several useful plant-derived proteins have already been identified. Plant cell culture would be particularly appropriate for the production of these "natural" therapeutic plant proteins, such as ribosome inactivating proteins (RIPs), which have a variety of potentially useful pharmaceutical activities such as antiviral and antitumor activity [2]. If production levels can be increased, plant cells may also be an important future source for the large-scale production of therapeutic proteins for human use.

## 2
## Foreign Protein Production in Plant Cells

### 2.1
### Background

The term "plant cell culture" refers to the propagation of any plant-derived cell tissue in gently agitated liquid media. Plant cultures can be classified as unorganized cultures (such as callus, suspension, or protoplast culture) or organized cultures (such as root or embryo cultures) depending on the tissue source and level of differentiation [3]. Cultures are first initiated by taking an explant from specific regions of a seed or plant and sterilizing it with hypochlorate or peroxide to minimize the risk of contamination. The sterile explant is treated with cell wall degrading enzymes, stimulated with hormones, and subsequently grown in medium (consisting of nutrients, salts, vitamins, and growth factors) until cell callus forms. After several generations, the callus becomes established (or friable) and may be transferred to liquid media where the cells will grow as a suspension. Initially these cultures grow slowly as large clumps of cells and must be cultivated in small flasks or six-well plates agitated by a gyratory shaker. After the new suspension has been subcultured into new media the cells become more evenly distributed and eventually develop into a fine suspension. Healthy, established plant suspension cultures can be easily cultivated in larger bioreactors. If desired, the cells can be treated again with cell wall degrading enzymes and grown as protoplasts. However, protoplasts have greater shear sensitivity and will regenerate a cell wall over time.

Beginning in the 1970s significant effort was invested to produce high-value secondary metabolites in plant cell culture. Despite the fact that some metabolites were produced at concentrations equal to or greater than those present in the corresponding plant tissues, only a few selected natural products could be produced economically in plant cells. The main limitation is the high capital costs of fermentation equipment compared to the moderate sale price and small market size for these substances. Around the same time, technology was developing for the manipulation of DNA and subsequent protein production in foreign host cells. With the successful marketing of early recombinant protein products such as insulin and human growth factor, it became clear that important and extremely valuable products could be produced using these methods.

For this reason, over the past two decades the production of recombinant proteins using plant cell culture has received considerable attention as a new research area. The feasibility of recombinant protein production also stems from the development of the *Agrobacterium* transformation technique [4] and other DNA-transfer methods, which incorporate the desired DNA into the plant genome. Plant cells may also be genetically transformed by several methods for the stable production of foreign proteins. The first transgenic plants were produced almost 20 years ago [5], followed closely by the first transgenic cell suspensions. These early studies focused on producing simple marker proteins or proteins from other plant species. Through further work, it became evident that producing commercially useful proteins with plant cells is a worthwhile goal because they can be grown on simple, protein-free media and are able to produce even the most complex protein products [6]. A significant number of proteins have already been produced on a laboratory scale by this method.

## 2.2 Growth Characteristics of Plant Cells

The growth pattern of plant cells in suspension culture is remarkably similar to the behavior of lower cell types such as bacteria and yeast. Figure 1 shows the typical growth performance for plant suspension cultures in terms of fresh weight and dry weight. These measurements (along with the settled cell volume and packed cell volume) are frequently used to monitor plant cell growth because counting individual cells within large aggregates to obtain a cell number is extremely difficult. The growth pattern of plant cells consists of three main phases: a lag phase, an exponential growth phase, and a stationary phase. There is also typically an acceleration phase and a deceleration phase separating these respective main phases. Doubling times for plant cell suspensions generally range from 1–4 days during exponential growth, depending on the cell type and culture conditions. These doubling rates correspond to total batch culture times of 7–28 days. Maximum cell concentrations typically range between 10 and 18 g $l^{-1}$ dry weight or 200 and 350 g $l^{-1}$ fresh weight.

Typically, the production of foreign proteins in plant cell culture is growth related, due to the use of unregulated strong promoters [7] such as the cauliflower mosaic virus (CaMV) 35S promoter. This strategy simplifies the optimization of protein production, because a reactor optimized for growth will likely also be near optimum conditions for product formation. As shown in Fig. 1, peak protein production is typically observed during exponential growth. The timing and level of maximal protein production are influenced by culture conditions such as cell concentration at inoculation and aeration [8]. Apparently, constitutive promoters are most active when the cell is actively dividing. However, this strategy of continuous protein production may burden the cells, increasing the likelihood of genetic drift or gene silencing. Also, the protein may be more susceptible to degradation, inhibition, instability, and other losses when it is produced gradually, since the average residence time for protein in the media will be several days. Inducible promoters can be used as an alternative to constitutive protein production. With this type of production

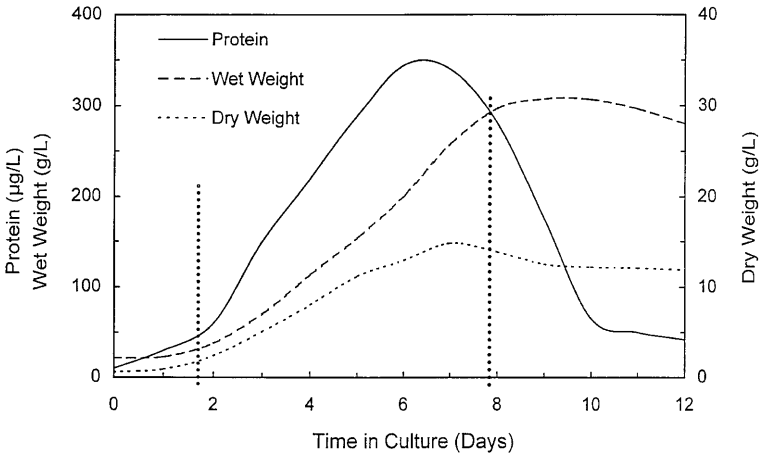



**Fig. 1.** Batch growth and antibody production of *Nicotiana tabacum* (BY-1) cells in terms of µg $l^{-1}$ protein, wet cell concentrations, and dry cell concentrations. *Dashed vertical lines* represent the boundaries between lag, exponential, and stationary growth phases. The 4-day offset between peak concentrations for biomass and protein product indicates that growth and product formation are not directly linked

scheme, cell growth and protein production steps are de-coupled. This production method can reduce the genetic burden to the transformed cells and the exposure of protein to degrading influences. These aspects of protein production will be discussed in more detail later in this chapter.

Unlike lower cell types, plant cells seem to effect changes in the pH of their growth environment during batch growth. In experiments conducted in our laboratory (unpublished results) attempts were made to overcome this effect by growing cells in media buffered with 0.1 M PIPES [piperazine-*N,N′*-bis(2-ethanesulfonic acid)] and by resetting the pH to 6.5 at the end of every day. Despite the increased regulation of pH in these latter two culture conditions, the pH tends to become more acidic during the exponential growth phase and less acidic during the deceleration and stationary phases. Although plant cell cultures are resistant to pH adjustment during batch culture, pH control is still possible through semi-continuous addition of acid or base in response to changes in pH. The need for pH control, and thus the effort that should be invested, will depend on the specific application. The option of pH control should be pursued in cases where the protein of interest is less stable under mildly acidic conditions or during semi-continuous protein harvest if the yield of the first purification step is sensitive to pH.

## 2.3 Product Range and Quality

A significant number of proteins have been successfully produced in plant cell suspensions. Table 1 presents a summary of important proteins along with re-

**Table 1.** Expression levels, activity, and quality of important proteins produced in plant cell culture

| Protein of interest | Expression level | Activity or function | Protein quality | Ref |
|---|---|---|---|---|
| Erythropoietin | 26 ng $g^{-1}$ total protein | *In vitro* erythroid colony formation | Homogeneous, glycosylated | [9] |
| Human Interleukin 2 | 0.10 mg $l^{-1}$ secreted | Cell proliferation | Not reported | [10] |
| Human Interleukin 4 | 0.18 mg $l^{-1}$ secreted | Cell proliferation | Heterogeneous | [10] |
| Guy's 13 murine monoclonal antibody | 18 μg $g^{-1}$ soluble protein | Growth inhibition of *Streptococcus mutans* | Heterogeneous | [11] |
| Human Granulocyte-Macrophage colony-stimulating factor | 0.25 mg $l^{-1}$ secreted | Cell proliferation | Heterogeneous | [12] |
| Anti-arsonate antibody (murine heavy chain) | 0.36 mg $l^{-1}$ secreted | Antigen matrix binding Protein G binding | Heterogeneous | [6] |
| Anti-TMV antibody (murine full-size) | 9 μg $g^{-1}$soluble protein | TMV binding | Homogeneous | [13] |
| Single-chain Fv ($\alpha$ phytochrome) | 5 μg $g^{-1}$soluble protein | Antigen binding | Homogeneous | [14] |
| Chloramphenical acyl transferase (CAT) | 17 units $ml^{-1}$ | Positive CAT assay | Not reported | [15] |
| Preproricin | 1 μg $g^{-1}$soluble protein | *In vitro* translation inhibition | Homogeneous, glycosylated dimer | [16] |
| Human $\alpha_1$-antitrypsin (AAT) | 22 mg $l^{-1}$ | Inhibition of porcine pancreatic elastase | Heterogeneous | [17] |

ported expression levels and the quality, activity, or characterization of the product. In every measurable case, plant-produced proteins demonstrate biological activity that is nearly indistinguishable or in a few cases higher than the activity of commercial standards. This tendency for good *in vitro* biological activity is an indication of proper folding, post-translational processing, and protein transport of the plant-produced protein. Despite the fact that plants and animals are from different biological kingdoms, their common status as "high" eukaryotes is reflected by their similar synthesis and processing of protein products.

Because of these findings, there is great optimism that plant proteins will exhibit *in vivo* activity as human therapeutic agents. However, some plant-produced proteins do exhibit a degree of heterogeneity in their molecular weight and chemical nature. In these instances, a few or even several different mass

species appear on Western blots of unpurified protein product. The mass differences are usually attributed to differing levels of glycosylation, product dimerization, or enzymatic cleavage. For use as an injectable therapeutic the protein product should be homogeneous after purification. Therefore, the source of this variation must be identified and eliminated. Otherwise, it may be necessary for one protein species (perhaps the one with the highest activity, largest concentration, or least risk of antigenicity) to be purified away from the others.

## 2.4 Product Secretion and Purification

One major advantage of plant cell culture as an expression system is the ability of plant cells to produce and secrete biologically active proteins. The term "protein secretion" refers to protein transport through both the plasma membrane and cell wall into the extracellular media. This is a metabolically dependent process [18] that can be directed by both native [6] and plant [14, 19] leader sequences. It is somewhat surprising that the native (mammalian) signal sequence is so effective in directing protein secretion in plant cells. One study suggests that protein secretion in plants follows a "default" pathway [20]. In this study, several non-secretory enzymes were directed for secretion using an endoplasmic reticulum (ER) directing signal. It may be that direction of the protein to the ER is necessary and sufficient to initiate a chain of events leading to protein secretion.

While the capacity of protein targeting and secretion is a hallmark trait of plant cells (along with other higher cell types), complete secretion (or export) of protein products beyond the cell wall and into the extracellular media has been problematic in some early and anecdotal cases. For example, plant-produced erythropoietin penetrated the plasma membrane of the cells but was confined within the cell wall during cell culture [9]. Following treatment with cell wall degrading enzymes, the protein was secreted at low levels during protoplast culture, due to the removal of the cell wall as an obstacle to secretion. Some efforts to secrete fully assembled antibodies from plant cells were also unsuccessful [21] with the assembled product also confined mainly to the apoplastic space. It has been postulated that proteins penetrate the plasma membrane by leader sequence directed export events and the cell wall by simple pore diffusion. As a corollary, it is hypothesized that penetration of the cell wall is the limiting step for protein secretion and that protein export is limited by the cell wall's pore size distribution for large proteins. Based on studies of cell wall structure the passage of large proteins through the cell wall into the extracellular media will be limited, perhaps with a cutoff as low as 20 kDa [22]. However, the structure and pore size distribution of plant cell walls varies for different species and even for different cell lines within a species. For cells that are well adapted to suspension culture, this cutoff appears to be significantly higher [6]. Protein transport through the cell wall may also be increased through treatment with dimethyl sulfoxide (DMSO) [23] or other permeabilizing chemicals.

If successful, transport of the protein out of the cell greatly simplifies downstream purification of the desired product because of the simple and protein-

free composition of plant culture media. Even at relatively low expression levels, the protein of interest comprises a significant percentage of the total secreted protein. Also, protein secretion eliminates the need for cell disruption. This omission reduces the difficulty of subsequent filtration or centrifugation steps because whole cells are simpler to process than cell debris. Since these steps are capital intensive, simplifying or eliminating them will significantly reduce purification costs. Despite these advantages, some aspects of protein recovery following secretion are still problematic. The low concentration of secreted protein in medium (due to limited protein expression levels) may increase the cost and difficulty of purification steps such as extraction and concentration.

## 2.5 Comparison with Alternate Host Systems

Microbial cells are excellent hosts for protein production because of their simplicity. Bacteria such as *Escherichia coli* grow rapidly (doubling time approximately 20 min) and accumulate high levels of protein (on the order of grams/l) without the product inhibition typical of other cell types. If every protein could be produced in an active form from microbial cells there might be no need for other hosts. However, the production of eukaryotic proteins in prokaryotic cells is limited because they lack the cellular machinery to fold, process, and secrete proteins. In general, eukaryotic gene products produced in prokaryotes are unfolded and inactive. Large quantities of unfolded protein accumulate as insoluble inclusion bodies that must be solubilized, refolded, and in some cases enzymatically treated to produce active protein. These steps are difficult, time consuming, and often have low yields. Even if they can be performed successfully, these steps greatly complicate protein purification leading to longer processing times and higher capital costs.

Some simple eukaryotic proteins can be produced using transgenic yeast cells. Protein yield from yeast cells is typically high – often in the range of 100 – 1000 mg $l^{-1}$. Commercial production equipment design and conditions for large-scale yeast culture are already well characterized by the beer industry. DNA can be transferred easily to yeast cells by well-established means using plasmid vectors or integrating vectors for small segments or yeast artificial chromosomes (YACs) for larger (> 100 kb) segments [7]. As eukaryotes, yeast cells are able to perform some post-translational functions that are impossible for bacterial cells. However, since yeast cells do not consistently remove introns, they are typically transfected with cDNA rather than natural genes. In addition, yeast cells carry out some post-translational modifications in a different manner than higher cells. Glycosylated groups on proteins produced by yeast are larger and contain more mannose than the correct patterns produced in mammalian cells. In some cases these enlarged and highly branched groups significantly hinder the desired activity of the protein of interest. Even in cases where *in vitro* activity is retained, *in vivo* activity may be lost due to systematic elimination of the foreign protein triggered by these antigenic differences in glycosylation. Eliminating potential glycosylation sites through point mutations in

the protein sequence can circumvent these problems. Since glycosylation is generally not required for biological activity, the modified protein will retain its desired activity. However, modifying the protein sequence to remove glycosylation sites is time consuming and labor intensive. Furthermore, the unglycosylated protein may differ significantly in its level of activity and stability *in vivo* compared with the native protein. Therefore, the most complex protein products (requiring glycosylation, assembly of multiple subunits, or other specialized modifications) may be better produced in "high" eukaryotic cells – cells originating from multicellular organisms.

For these "high" eukaryotic proteins, the most widely and commonly used host systems are mammalian cell culture and insect (or baculovirus based) cell culture. Mammalian cell culture produces the highest protein yields of these options – often in the range of 10 – 50 mg $l^{-1}$. Traditional mammalian cells, such as baby hamster kidney cells (BHK), are anchorage dependent or shear sensitive and must be cultured in flask-type growth chambers or on microcarriers in complex and expensive serum-containing media. These growth conditions result in slow growth and product that is difficult to purify from contaminating protein in the media. Newer and more robust cell lines, such as protein-free Chinese hamster ovarian cells (PF CHO) or hybridoma, have been successfully cultivated in spinner flasks and serum-free media. However, acclimation of mammalian cell lines to protein-free media requires several passages and careful attention. Since spinner flasks are inadequate for culture volumes beyond a few liters, large-scale cultivation of mammalian cells is typically limited to expensive perfusion or membrane reactor systems. Despite these challenges, the efficacy and appropriateness of commercial protein production using mammalian cells are well established for antibodies and other naturally occurring protein products. Insect cell culture produces lower protein yields than mammalian culture – typically in the range of 1 – 10 mg $l^{-1}$ – but it is a more rapid and flexible production system. Through the use of the *Autographa californica* polyhedrosis virus, baculoviral constructs can be constructed by replacing the polyhedrin viral coat gene with a gene for the protein of interest [7]. Using this modified virus, untransformed insect cells can be directly induced to produce a protein of interest through the simple mechanism of viral infection. Since the viral DNA carries the foreign gene of interest, production levels for this system are very stable and predictable provided that infection conditions (and viral stock characteristics) remain constant. The major disadvantage of baculoviral expression is the destruction of insect cells during the course of infection. Cell lysis, a natural consequence of viral infection, ends the reproductive potential of the production culture and releases contaminating protein into the media along with the desired product. In comparison to these more established systems, plant cells express significantly lower levels of protein than prokaryotic, yeast, mammalian and even insect cell based production. This difference is mitigated by lower media costs, simpler purification, and the lack of human pathogens in the plant cell system. Given a complete economic analysis of both protein production and purification, plant cell culture may be competitive with these more established systems in some cases. Obviously, if protein yields for plant cell culture were closer to mammalian cell production levels, this would

be the preferred mode of production for proteins that are not amenable to production in yeast or bacterial hosts.

### 2.6 Obstacles and Limitations

At present the predominant obstacle preventing the commercial implementation of plant cell culture for foreign protein production is low expression levels. Since protein production in plant cells is a new technology, there is cause for optimism. Several of the following sections will highlight new approaches that may lead to significant increases in protein productivity. There is also some question about the exact chemical nature of glycosylation groups added by plant cells. While studies indicate that plant-produced products are biologically active *in vitro*, studies of the *in vivo* activity and possible immunogenic behavior of plant-produced proteins have been scarce. The techniques needed to definitively answer these questions about protein structure, behavior, and activity require larger quantities of purified material than the amounts typically produced in plant cell experiments. Increased protein expression, increased reactor scale, and full purification of plant-produced proteins should provide answers to these important questions. There are also some unresolved problems concerning the homogeneity of plant-produced proteins. Western blot analysis reveals multiple forms of plant proteins, which may be problematic for the application of plant-produced proteins as injectable pharmaceuticals. Other limiting factors include longer times needed to produce and isolate stable transgenic plant cell clones (because of slower doubling times) and the lack of dependable cryopreservation protocols for plant cell suspensions. These unsolved problems should be viewed as challenges that, if overcome, will provide insights toward the improvement of all protein production systems.

## 3 Foreign Protein Production in Plants

### 3.1 Background

"Plant-based" protein production refers to the production of a protein of interest in whole, intact plants (or plantlets) as opposed to undifferentiated plant tissue. The stable transfer of foreign DNA to the main genome of plants dates back to 1983 [24]. Since that time, the transfer of single or multiple genes to plants has been utilized primarily to confer pathogen resistance or other favorable agricultural traits. In these studies the whole plant (or a specific organ, fruit, or cereal) was the desired product and genetic modifications were used to improve the quality, performance, or robustness of the plant. The production of specific genes as products in whole plants began in 1989 with the production of antibodies in tobacco leaves [25]. Subsequent studies have produced a wide variety of proteins (from $\alpha$-amylase to xylanase) with a wide range of activities and sizes [5]. In these studies the desired product is large, concentrated amounts of

the single recombinant protein rather than plant biomass for its own sake. While productivity varies for different products and host species, reported production levels are typically near 1% of the total protein in the harvested plant or tissue. In most cases, the absolute amount of protein is not reported so it is difficult to compare these expression levels with other production methods.

The production of foreign proteins in whole plants is a more advanced field than protein production in plant cell culture. In fact, several products are under development by companies such as Integrated Protein Technologies (a unit of Monsanto) [26], Applied Phytologics [27], and Prodigene, Inc. [5, 28]. Protein production in whole plants is particularly appropriate in cases where a plant tissue such as a fruit, tuber, etc. can be used to deliver the protein of interest. For example, oral vaccines are currently being developed in potatoes and other edible vegetables [29]. Protein production using established field crops is inexpensive and extremely scalable using current agricultural methods. The screening and preservation of good clones (or transgenic "cultivars") may also be accomplished through established agricultural means.

### 3.2
### Product Range and Quality

In general, plant-produced proteins are correctly folded, glycosylated, homogeneous, and biologically active [5]. In several cases, their activity has been shown to be nearly indistinguishable from their mammalian counterparts [30]. This is almost certainly due to the commonalties in protein synthesis, folding, and post-translational modification pathways shared by plant and mammalian cells. To date hundreds of proteins, including more than 30 distinct types with direct economic value [5], have been produced in plants. These numbers continue to climb steadily as new research accumulates in this area. As mentioned above, these proteins span a wide range of source species and activities. One unique characteristic of protein production in whole plants is their natural capacity for long-term protein storage. Recombinant proteins confined within the leaves or seeds of plants may be stored for several weeks, months or perhaps years with only minimal losses in protein activity [30]. Whole plants are recognized as an excellent potential source of recombinant antibodies [31]. That is why, as mentioned above, several companies are advancing plant-produced products toward FDA approval and marketing.

### 3.3
### Obstacles and Limitations

Despite this area's promise there are some obstacles and limitations that must be solved, or at least considered, as plant-based proteins move toward the marketplace. The most intrinsic drawback for protein production in plants is time. When a protein is expressed in a whole plant, its production is linked directly to the development of that plant, often from seedling (or plantlet) to mature plant. In cell culture techniques, where cells are growing at maximal rates in an artificial environment, the time scale for producing a batch of protein is on the

order of days. For protein production in plants, where plant tissue is cultivated during the course of a natural growing season (or perhaps in a greenhouse environment), the time scale for producing a batch of protein is on the order of months. This difference in time scale is also present during the initial regeneration of transformed plantlets and the selection of high producing plant clones. This disadvantage is mitigated by the enormous amount of biomass (and accompanying protein) that may be produced in an "agricultural" sized batch.

Another potential disadvantage for protein production in whole plants is gene inactivation. Cell cultures are often stable for long-term protein production, especially with the periodic use of selecting agents or recurrent use of low passage cell stocks. In contrast, transgenic plants may lose their ability to produce protein product due to gene-silencing events. Incidences of gene inactivation are correlated with several factors, including high protein expression levels, repetitive homologous promoter sequences, repetitive homologous coding sequences, and multiple copy gene integration [5]. A high incidence of gene-silencing events in the field could prove economically deleterious because of energy wasted on harvesting and processing crops that contain little or no protein. Field contamination with non-transgenic or closely related crops would pose a similar economic threat.

The most important limiting factor for protein production in whole plants may be purification. Recombinant protein expression levels in whole plants are typically quite high. However, even for "secreted" products, this protein will be inevitably trapped within the plant's tissue. In some cases protein may be recovered through direct extraction, which may supply inadequate yields, or tissue infiltration [32], which is likely not to be scalable. Therefore, the first purification steps in most cases will be plant (or organ) harvest, tissue homogenization, protein extraction (if appropriate) and clarification by filtration or centrifugation [5]. These steps are capital intensive and result in contamination of the plant product with protein impurities (natural proteins from the host tissue) and degrading influences such as proteases, phenolics, and (in the worst case) shear and foaming.

### 3.4 Comparison with Production in Plant Cells

In reality, the technologies of protein production in plant cells and in whole plants are almost inextricably linked. Transgenic plants are typically regenerated from genetically modified tissues or suspensions and suspension cell lines may be derived from transgenic plants [30]. The two technologies may be viewed as two variations on the same theme, each with their own strengths and weaknesses. Production in whole plants tends to give higher protein yields, but this occurs at the cost of longer cultivation times and a more difficult purification task. Similarly, secreted proteins are more easily recovered and purified from suspension cultures, but they tend to accumulate at lower levels. Even active researchers working in the area of protein production in whole plants recognize the advantages of using cell suspensions [33]. Their well-defined growth conditions, controlled sterility, and simpler purification methods miti-

gate the lower protein expression levels. For example, the production of ricin (a potential anticancer agent) by cultured tobacco cells led to more consistent yields and more favorable processing [16] than its production in whole tobacco plants [34]. As these technologies mature, it is likely that they will continue to compliment one another well.

## 4
## Gene-Transfer Techniques

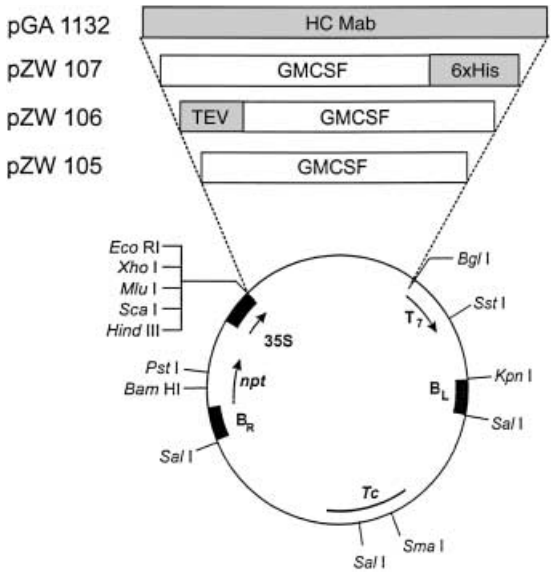
The construction and transfer of DNA to plant tissue is a critical step for successful protein production in plant cells. Decisions made during the planning and execution of this phase will affect not only direct considerations such as the transformation efficiency and the difficulty in selecting clones, but also indirect considerations such as the ultimate performance of the cells and production level of the final product. Bearing this in mind it is not surprising that some of the most important intellectual property in the realm of protein production in plant cells falls into this category. Care must be taken in selecting the elements of the transfer DNA, the method of DNA transfer, and the strategy that will be used for clonal selection. In an informative review article, Birch suggests that the essential requirements for a gene-transfer system are a source of compatible target tissue, a method of DNA introduction, and a procedure for selecting and cultivating transformed cells [35]. To this list we would add developing an appropriate DNA construct as an additional required element. The essentials of these four tasks will be discussed in the sections that follow.

### 4.1
### Target Tissue for DNA Transfer

For the transformation of suspension cells, a wide range of cell types is available. The most widely used cell type is tobacco, particularly the BY1 and BY2 cell lines derived from *Nicotiana tabacum*. These cell lines have excellent growth characteristics and are easily transformed by co-culture with *Agrobacterium* (discussed in more detail below). In general, dicotyledons, such as tobacco, have excellent growth characteristics and are easily transformed while monocotyledons, such as corn, are often less robust in culture and are more difficult to transform. However, since some studies indicate that plant species carry out different degrees of post-translational modification and levels of expression [26], the transformation of both monocotyledons and dicotyledons or of several alternative species in parallel may be advantageous. Beyond these considerations, candidate tissue for transformation should have favorable growth characteristics and should be pathogen free.

### 4.2
### Construction of Transfer DNA

The construct requirements for the transfer of DNA to plant cells vary depending on the transfer method. Obviously, the foreign DNA must be genetically compatible with the plant cell host and there must be some means of differen-




**Scheme 1.** Design elements of a typical plant cell expression vector, including promoter sequence (*35S*), terminator sequence (*T7*), resistance gene (*npt*), right and left borders (*Br* and *Bl*), and insertion point for genes of interest. This binary vector is suitable for plant and *Agrobacterium* host cells

tiating between transformed and untransformed tissue. Therefore, at minimum, the transgene must include an effective promoter sequence, the gene of interest, a terminator sequence, and a selectable marker. Scheme 1 shows a typical plant cell expression vector.

For *Agrobacterium* transformation, the gene of interest must be carried in a binary vector with appropriate bacterial T-DNA elements. For other transformation methods the requirements may be less stringent. However, even in these cases, the DNA of interest is typically amplified in bacteria. The choice of a promoter is of particular importance, as this element will dictate both the expression level and the time course of protein expression. The inclusion of additional elements, such as enhancers, 5′ and 3′ untranslated regions, and fusion elements (e.g. fluorescent or 6-histidine tags), should also be considered for improved expression, detection, and downstream processing.

## 4.3 DNA-Transfer Methods

### 4.3.1 *Agrobacterium Transfer*

The transfer of foreign DNA to plant tissue material is most commonly achieved using the natural gene transfer function of *Agrobacterium tumefaciens*, a "parasitic" soil bacterium.

*Agrobacterium* utilizes a highly adapted gene-transfer and integration technique to integrate single-stranded DNA into plant cells [35]. The mechanism for this transfer is not well understood. However, this method is widely used since it gives predictable results. *Agrobacterium* transformation produces genetically transformed plant cells or tissue that is highly stable [36] because the foreign gene is inserted into the main plant genome. The typical procedure for *Agrobacterium*-mediated transformation includes co-culture of plant cells with the bacteria, antibiotic treatment to disinfect the culture and kill the contaminating bacteria, selection of transformed plant cells using the conferred resistance trait, and identification of high producing clones through callus culture under continued selection pressure. In all, this procedure can yield stable clones for further research in approximately 2 months for cells co-cultured with *Agrobacterium* and 1 year for leaf disk transformation [33].

### 4.3.2 *Microprojectile Transfer*

Microprojectile bombardment (or BIOLISTIC transformation) is also a common and effective method of DNA transfer. In this method, gold microcarriers are coated with plasmid DNA and delivered to plant tissue by means of a particle gun. At the time of particle delivery, DNA enters the cell by mechanical means and, in a small percentage of events, is able to incorporate into the plant genome. It is likely that particle delivery also damages the host DNA by shearing. Such a disruption would give the opportunity for foreign gene incorporation by the plant's DNA repair mechanisms. Damage to the host DNA also places the viability of the tissue at risk. Because of damage caused by bombardment, the newly transformed plant tissue is initially fragile and must be cultivated under selection-free conditions until signs of visible growth are observed. Because of its basis on mechanical delivery, microprojectile transformation can be used to deliver multiple plasmids simultaneously [37]. However, to achieve balanced expression of multiple genes, it is advisable that each separate plasmid carries a unique selectable marker. The major advantage of this method is wide applicability – even some of the most "recalcitrant" species can be transformed [35]. The main disadvantages of this method are low transformation efficiency, DNA fragmentation, and collateral genetic damage.

### 4.3.3 *Other Methods*

Between the methods of *Agrobacterium* and microprojectile transfer, nearly every plant species can be transformed effectively [35]. However, these methods are covered by patent claims and may result in limited transformation efficiency for some cell types. For this reason, the use of alternative gene-transfer methods is an active area of research for all cell types. Alternative gene-transfer techniques include electroporation, microinjection, liposome fusion, direct transfer into protoplasts, and laser treatment [38]. In electroporation, DNA is transferred into the cell using a high-voltage electrical pulse [39]. Standard

techniques for the production of transgenic plant cells by electroporation often include a pretreatment with cell wall degrading enzymes [40]. In general, electroporation yields a high transfection frequency and stable integration of the desired transgene. Microinjection utilizes a microscopic delivery system to directly inject genetic material into host tissues. Since this method is exacting and requires specialized equipment it is generally avoided.

New research efforts also include transient protein expression in plant cells using viral expression systems. Essentially, this strategy mimics the pattern of baculoviral expression using insect cell culture. A natural virus infects the cell, usurps the cellular machinery to reproduce its viral components, and causes cell lysis to distribute and propagate the new viral particles. By infecting cultured plant cells with a virus carrying the gene for the protein of interest, production of the desired protein product will accompany the natural course of infection. Transient (virally induced) expression generally has the advantage of higher protein expression levels (in terms of protein concentration per liter), but the disadvantage of a limited production interval and destruction of the host cells. A discussion of the implications for transient protein production in plant cells will be included in our discussion of new schemes for protein production.

## 4.4 Cell Selection and Screening

Following DNA transfer, transformed plant cells must be selected and, subsequently, high producing cell lines must be identified. The effectiveness of this selection and screening procedure is an important aspect of successful protein production. For genetic stability, it is critical that true plant cell clones are isolated. For good productivity it is critical that the highest producing cells are identified from among the full distribution of possible clones. At the conclusion of transfection, the plant cells exist as a mixture of transformed and untransformed cells. In the case of *Agrobacterium* transformation the cells are co-cultured with transgenic bacteria also containing the selectable marker. In this case, the culture must be decontaminated by the use of cefotaxime or other microbe-specific antibiotic agents.

The selection of transformed cells is achieved using a conferred selectable trait, most commonly antibiotic resistance. Only transformed cells with the conferred resistance trait are able to survive and reproduce. However, the use of antibiotics as a media component for commercial protein production is problematic for at least two reasons. First, residual antibiotic would be a highly undesirable contaminant in the final product. Second, the recurrent use of antibiotics increases the likelihood that antibiotic-resistant pathogen strains will develop. Recent studies indicate that antibiotic selection is not necessary during every passage (or generation).

An interesting alternative method for the selection of transgenic plant cells utilizes the principle of "positive selection" [41]. This method uses a novel glucuronide cytokinin derivative as a selective agent and the *E. coli* $\beta$-glucuronidase gene (GUS) as the selectable gene. Only transformed cells carrying the GUS gene are capable of cleaving the cytokinin into its active form. Since plant

cells cannot grow without stimulation from cytokinins or other hormones, only transformed cells will be stimulated to grow. A similar new method of selection is based on the use of phosphomannose isomerase (PMI) as a selectable marker [42]. Normally, plant cells cannot metabolize mannose as a carbon source. However, transformed plant cells with the PMI gene can convert mannose to fructose and then metabolize that carbon source by natural pathways. Untransformed plant cells will not grow due to carbon starvation.

Regardless of the method, the goal of selection is to eliminate untransformed cells, restricting the population to those with potential to produce the protein of interest. Following selection, a screening (or cloning) strategy must be used to isolate high producers from among the full population of cells. Because most DNA-transfer strategies insert the gene of interest in a "random" location, the gene of interest will be transcribed at different frequencies (and even at different periods of the cell cycle) corresponding to the genomic location of the point of insertion [12]. At the level of translation, the population of cells will produce corresponding levels of protein. Screening is most commonly accomplished by plating a low density of selected cells onto solid selective media so that distinct "clones" of callus form on the plate. Once they are established, these calli are transferred to fresh plates and assayed for protein production levels by ELISA, Western blotting, or other methods. Fusion of the gene of interest with a reporter element such as green fluorescent protein (GFP) or other easily detectable markers can greatly simplify the identification of transformed plant cells and the quantification of the desired product [43]. However, for many applications, this fusion element must be removed during product purification. Once the protein of interest has been quantified, the cells exhibiting the highest levels of protein expression are isolated and transferred to liquid media.

## 5
## Increasing Protein Production in Plant Cell Culture

### 5.1
### A Multistep View of Protein Production

In simple organisms it is somewhat safe and effective to focus optimization efforts on just a few or even one area to affect a desired improvement in productivity. For example, to increase protein production in *E. coli* it may be sufficient to increase the plasmid copy number or to add a chaperone or folding aid. However, in higher organisms, changes in behavior due to these types of strategies are not as predictable or easy to control. This is due in part to the fact that there are several additional steps along the protein production "pathway" that are unique to higher cells. The successful production of a protein from any higher cell type is a multistep process, requiring efficient transcription, post-transcriptional processing, translation, post-translational processing, and secretion [5]. Any of these may be a limiting step for protein production in a given system. The present body of research does not suggest a single limiting step or "bottleneck" for plant cell systems in general. In fact, significant improvements in protein expression and recovery have resulted from work on

several of these fronts. These include, increased translation using the tobacco etch virus 5′ untranslated region (TEV UTR) [12], secretion directed by native [10] or plant signal sequences [14, 19] in conjunction with exogenous stabilizers [6], transcription using scaffold attachment regions (SARs) [44], or even novel procedures in isolating clones (unpublished results). From this information one can conclude either that the limiting step varies depending on the protein of interest, or that many steps in the protein expression cascade must be optimized. Bearing this in mind, each step along the protein production pathway may be viewed as a frontier for new research.

## 5.2 Instability of Protein Products

In recent years, increasing attention has been directed toward protein stability problems as a target area for increasing the yield of protein products. The degradation, or loss, of protein product after synthesis may occur by a number of mechanisms; including enzymatic breakdown, aggregation, denaturation, and adsorption. Regardless of the cause, any event that significantly reduces the amount of functional or active protein constitutes a costly loss of product. In general, protein instability is caused by unfavorable interactions between the protein and its chemical environment. As such, it can be addressed either by modifying the structure of the protein or by modifying its chemical environment. Several studies have noted that the plant growth medium is not an ideal environment for dilute concentrations of recombinant protein. As result, work in our group and other laboratories has focused on modifying growth media with additives to increase the stability of secreted proteins.

## 5.3 Methods To Enhance Protein Stability and Production

Due to the occurrence of unfavorable environmental conditions, the loss of protein due to product instability during plant cell culture and subsequent purification significantly influences product yields. Protein stability following secretion can be improved with the addition of appropriate chemical agents (or stabilizers) to the growth media or storage solution. Previous work in our laboratory demonstrates increased recovery of a mouse monoclonal antibody heavy chain by the addition of DMSO [45], gelatin [46] and polyvinyl pyrrolidone (PVP) [6, 47]. As shown in Fig. 2, the level of secreted protein is significantly increased with the addition of these exogenous stabilizers.

Similar results were observed for the stabilization of an antibody produced in hairy root culture [11]. In contrast, some proteins are more resistant to stabilization efforts. As shown in Fig. 3, plant-produced granulocyte-macrophage colony-stimulating factor (GM-CSF) and Interleukin 4 (IL-4) are only moderately stabilized with the addition of bovine serum albumin (BSA). The use of known stabilizers such as PVP and gelatin was ineffective for these proteins. This difference highlights the need for more general protein stabilizers for use in plant cell cultures.

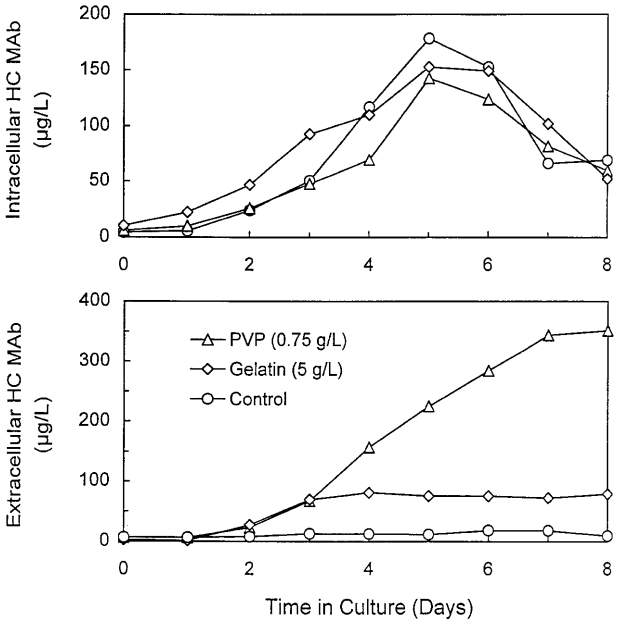



**Fig. 2.** Effect of exogenous gelatin [46] and PVP [47] on the production and stability of a murine antibody heavy chain. *Triangular symbols* indicate PVP, *diamonds* indicate gelatin, and *circles* indicate no stabilizer (control). In the upper portion of the figure, the intracellular protein is unaffected by the addition of exogenous stabilizers. In the lower portion of the figure, added PVP and gelatin significantly increase the level of extracelluar protein product

One interesting new stabilizer that may help fulfill this need is the protein bacitracin, which has been shown to enhance cell growth [48] and inhibit the degradation of plant-produced proteins [49]. The use of stabilizers to increase protein stability is not exclusively limited to plant cell culture. Other published examples include the stabilization of acidic fibroblast growth factor by nucleotides [50], malate dehydrogenase by sulfobetaines [51], and ribonuclease A by sorbitol [52]. By using this strategy, protein yields can be increased dramatically with the addition of inexpensive chemical agents. However, in the ideal case, the stabilizing agent should not complicate downstream purification of the product.

## 5.4
## New Schemes for Protein Production

Even with the use of stabilizing agents and "optimized" transfer DNA, research in our laboratory suggests that there is a maximum protein yield for plant cells using standard production conditions [12]. Given this apparent limitation, the most promising new methods seek to increase protein production by devising new production strategies that will overcome product instability and inhibition or that will increase the rate or level of protein expression. Emerging schemes include the use of new promoter sequences, semi-continuous protein harvest to

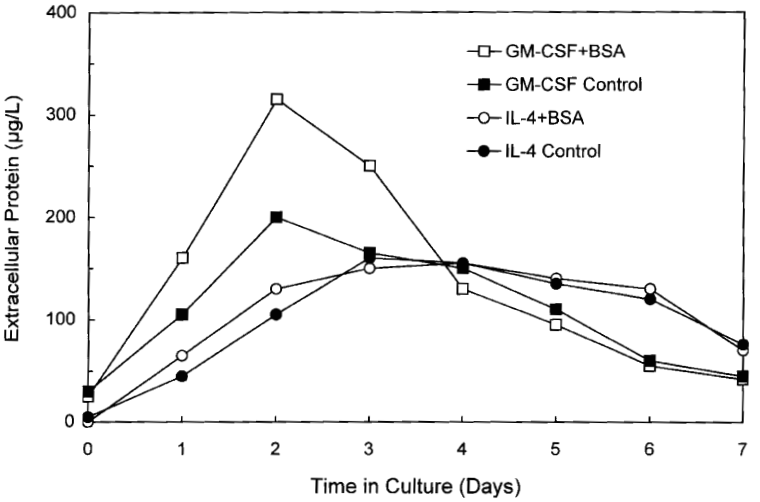



**Fig. 3.** Effect of exogenous BSA on the production and stability of plant-produced human GM-CSF [12] and IL-4 [10]. *Square symbols* represent GM-CSF production and *circles* represent IL-4 production. *Open symbols* indicate added BSA. Sterilized BSA was added to media prior to inoculation at a concentration of 100 mg $l^{-1}$ For GM-CSF, adding BSA increases the level of extracellular protein. For IL-4 there is no significant effect

eliminate protein stability or inhibition problems, and virally induced protein production. Each of these novel methods seeks to circumvent the traditional limitations of protein production in plant cells by changing the behavior of the system.

### 5.4.1 Inducible Promoter Systems

Several new expression strategies utilize inducible promoter systems in an effort to de-couple cell growth and protein production. This is a potentially powerful production scheme, especially if it can be combined with cell immobilization (discussed below). Unlike constitutive promoters, which are always active, inducible promoters are triggered by a specific stimulus. This function can be the result of natural promoter sequences or, in certain circumstances, it may be conferred through the use of paired repressor and activator proteins in a scheme mimicking bacterial operon systems such as the lac operon. A wide variety of inducible plant promoter systems appear in the literature including steroid [53], auxin [54], heat shock [55], metal [56], tetracycline [57], salt [58], sugar starvation [59], and ethanol [60] inducible systems. Comparison of different inducible systems indicates that each promoter stimulates different levels of protein production under inducing and baseline levels of the induction agent – even after optimization of induction conditions [61]. Typically, an inducible promoter is chosen to give "tight" control and "strong" promotion of the gene of interest. This means there should be low or negligible protein production

rates under baseline conditions and high protein production rates under induction conditions.

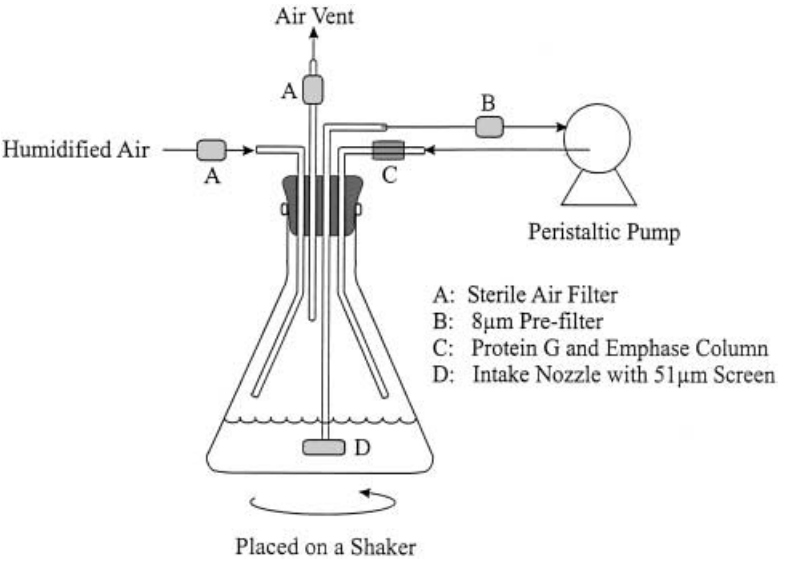
Since inducible promoters are triggered through the addition (or removal) of exogenous chemicals or through changes in environmental or growth conditions, their activation is independent of cell growth. Instead, protein production is triggered at a designed growth stage or even in a separate reactor. In general, the goal of inducible protein production is to stimulate a large burst of production once the cells have reached the determined optimum biomass concentration or growth phase. Using standard suspension culture this strategy requires several unproductive days of cell growth before protein can be produced and, in some cases, a medium exchange to rapidly expose the cells to induction conditions. If immobilized cells are used, beads or carriers can be pre-made with cell concentrations at or near the ideal conditions and then reused several times. Also, since the cells are trapped inside the immobilizing matrix, media replacement proceeds quickly and easily once the beads or carriers have settled.

Inducible protein production may also have advantages over constitutive expression in the area of protein stability. Much of the instability of secreted proteins can be attributed to the harsh, dilute environment of the extracellular media. Although the mechanism of protein loss is unknown, it is clearly a time-dependent process [6]. With the inducible promoter system similar (or larger) amounts are produced over a very short amount of time. This "compressed" production interval allows less time for time-dependent protein loss and leads to a less dilute protein environment even in the absence of stabilizers.

### 5.4.2
### *Affinity Chromatography Bioreactor*

As discussed earlier, secreted proteins are often unstable in growth media. This behavior, coupled with mechanisms for product inhibition, deactivation, and proteolysis, significantly decreases the yield of recoverable proteins for traditional bioreactor configurations. If the desired product could be harvested before it is degraded, and if product inhibition effects were minimized, protein yields should increase significantly. A novel strategy employed in our laboratory is the use of an affinity chromatography bioreactor or ACBR to simultaneously produce and harvest recombinant proteins from plant cell culture. The features of the ACBR (Scheme 2) include a cell growth chamber where protein is produced, a cell retention screen allowing separation of biomass from the protein harvest stream, an affinity chromatography column used to collect the protein of interest, and a media return loop which returns media to the growth chamber.

Reactors of this type have been used to produce and harvest the heavy chain of a murine monoclonal antibody using protein G affinity and histidine-tagged human granulocyte-macrophage colony-stimulating factor (GM-CSF) using metal affinity chromatography. Implementation of this scheme on a small scale was difficult, but these studies show that the amount of recoverable protein can be increased [62]. As shown in Fig. 4, recoveries of human GM-CSF and a mouse monoclonal antibody heavy chain were increased by three- and seven-fold, re-




**Scheme 2.** Affinity chromatography bioreactor (ACBR) for the semi-continuous recovery of plant-produced proteins. Media is drawn up through the intake nozzle (*D*) during culture, through a pre-filter (*B*) that removes particulates, and then to a protein-collection column (*C*) using a peristaltic pump. After protein binding, the media is returned to the growth chamber. Humidified air is supplied to the culture through a sterile pre-filter (*A*) [62]

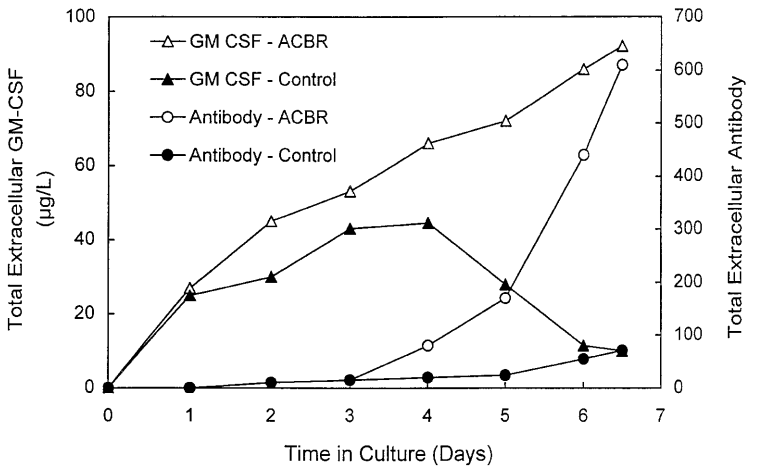



**Fig. 4.** Increased recovery of plant-produced GM-CSF and murine antibody in the affinity chromatography bioreactor (ACBR). *Triangular symbols* represent GM-CSF production and *circles* represent antibody production. *Closed symbols* indicate normal batch production and *open symbols* indicate ACBR production. For both products, protein recovery is increased several-fold using the ACBR [62]

spectively, as compared to normal batch production. This improvement is actually considerably less than the expected increase. This shortfall is due in part to the short recirculation times used in these experiments and in part to inadequate performance of the chromatography columns over repeated semi-continuous use.

Studies using this strategy clearly demonstrate that product inhibition is a significant limiting factor for protein production in plant cell culture. It is possible that, on a larger scale, reactor configurations of this type will lead to more significant increases in protein yield from plant cell cultures.

### 5.4.3
### *Transient Protein Production*

Another new method for protein production utilizes plant viruses to stimulate transient protein expression in plant cells. Virally based protein production constitutes both a novel method of DNA transfer (as discussed above) and a novel production scheme. This strategy is essentially the same as baculoviral infection of insect cell cultures for transiently induced protein expression. First, the gene of interest is cloned into the viral genome under the control of a judiciously chosen promoter sequence. This insertion may be accomplished by replacement of a nonessential viral gene, insertion of an independent strong promoter, or fusion with an existing viral gene product [63]. Subsequently, the virus is amplified (using a small-scale infection) to produce a working viral stock and administered to plant tissue for protein production. Viral expression strategies have recently been developed or modified for plant tissue using cauliflower mosaic virus [64], maize streak virus [65], and numerous others. At present, virally driven protein production strategies have been used almost exclusively in whole plants to conduct basic research in plant biology and pathology. More recently, the production of antigenic peptides (epitope presentation) using plant viruses has been investigated for the development of oral vaccines [66]. Further development of these methods for the production of commercially useful protein products and application of virally induced production to plant cell cultures may give impressive results.

## 5.5
## Modeling Considerations

Particularly as protein production schemes become more complex, intuition and experience will be insufficient to predict appropriate conditions and understand the observed behavior for the production of a protein of interest. Because of the large number of variables, the wide range of possible values for these variables, and the long time-scale for experiments, it will also be nearly impossible to determine optimal production conditions through experimental data alone. Any efforts to understand and optimize plant cell culture behavior can be aided greatly with the use of mathematical modeling. In general, a model can be used for two distinct purposes: either to explain the measured behavior of a given system, leading to theoretical mechanisms for the observed

behavior, or to predict the behavior of the system leading to estimates of system performance over a wide range of conditions. Both of these aspects of mathematical modeling are useful for studying plant cell cultures. Early in model development, a basic modeling approach must be chosen.

Traditional models range from simple (unstructured and unsegregated) to very complex (structured and segregated). The full implications of these choices, and subsequent model development, are too involved to cover here in any depth. However, the reader is cautioned that in most cases unstructured models have proved inadequate to describe the complex behavior of plant cell cultures. Structured kinetic modeling, first applied to plant cell cultures by Frazier [67], more adequately describes cell growth (including the lag phase), cell viability, and product synthesis. At present, few researchers have undertaken full modeling efforts to describe protein production in plant cell culture, but the effort can be fruitful. An approach developed in our group successfully modeled plant cell growth, protein production, secretion, degradation, and interactions with exogenous stabilizer molecules [68]. With the implementation of novel production schemes, as described above, modeling approaches will be essential for process optimization.

## 6
## Scale-Up Considerations

### 6.1
### Demand for Alternative Hosts

During the early developmental stages of biotechnology, companies tended to develop expertise for expression in a single host system. More recently, it has become clear that individual proteins are expressed more efficiently, secreted more easily, or perhaps folded and modified more consistently in a given host as compared to other alternative hosts. Following this reasoning the ideal host for expressing a protein of interest will change from case to case depending on issues such as codon usage, difficulty of folding, product homogeneity, and protein modifications. As a result, the biotechnology industry is becoming increasingly open to the idea of comparing alternative hosts for the production of promising therapeutic agents. Legal issues such as intellectual property, patent rights, and licensing agreements also make the development of novel host systems a favorable choice. With continued development, plant cell culture should become a more common candidate host system. The successful application of plant cells as production lines in the commercial arena will, in turn, necessitate cultivation in large bioreactors.

### 6.2
### Plant Cell Bioreactors

For initial studies, plant cell suspensions are typically cultivated in shake flasks agitated by a gyratory shaker, but for the economical production of commercial products, plant suspension cultures must be cultivated in larger bioreactors.

Such reactors are essentially the same as those used for microbial culture [69]. However, the design of suitable bioreactors for plant cell culture also presents some unique challenges because plant cells tend to form large aggregates. Concerns for the scale-up of plant cell bioreactors include shear sensitivity, high viscosity, and risk of contamination. The problem of foaming, once a significant concern, can be mitigated through the addition of small quantities of mineral oil or other antifoaming agents to the reaction vessel [70]. Experimental studies [71] show that plant suspension rheology is a function of cell size and morphology and can be controlled. Traditional plant bioreactors use stirred tank, airlift reactor, or similar configurations. New approaches include the use of membrane stirrers for improved aeration [72]. Plant cells may also be amenable to culturing in hollow fiber or perfusion reactors. For protein expression applications, plant cell bioreactors must be configured and optimized not only for cell growth but also for product formation. The use of bioreactors for large-scale protein production is a relatively new application, but knowledge derived from the production of natural products from plant suspensions will be easily transferred to this new application.

## 6.3 Cell Immobilization

Plant cell immobilization is an important process alternative for plant cell cultivation. The use of encapsulating gels, such as alginate, to immobilize plant cells is a relatively old technology dating back more than 20 years [73]. It is well known that cell immobilization protects the cells, facilitates the re-use of cells in continuous or semi-continuous culture, and allows higher inoculum percentages than standard methodologies. A significant body of work in the literature has shown that cell encapsulation has a number of favorable effects. The changes in growth environment of the cells can significantly influence the product yield for natural plant products [74]. These encapsulated cells are also less sensitive to shear stresses because of protection from the surrounding gel material. Gel beads have more favorable settling velocities than cells because their diameter is much larger than the individual clumps of cells.

Procedures for plant cell immobilization are represented in the literature for a wide variety of encapsulating agents. These include alginate, agarose, gelatin, carrageenan, polyacrylamide, and combinations of these substances [75]. To form spherical beads, mature (late exponential or early lag phase) cells are mixed with the encapsulating agent until a homogeneous slurry is formed and transferred drop-wise into the gelling reservoir. For most thermogels, this method is not suitable because exposure to high temperatures will significantly reduce cell viability. Instead, the slurry may be rapidly dispersed in a two-phase oil/water system. While this dispersion method allows the use of a wider range of encapsulating materials, it produces a heterogeneous mixture of bead diameters. Recent studies in our laboratory also indicate that plant cell immobilization can lead to increased protein production [76]. As shown in Fig. 5, merely immobilizing the plant cells in alginate beads can increase the production of human GM-CSF.

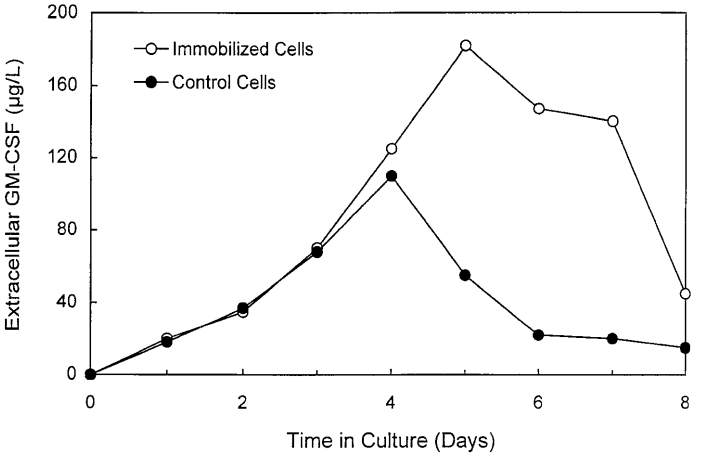



**Fig. 5.** Increased expression of human GM-CSF by immobilized plant cells [76]. *Closed symbols* indicate normal batch production and *open symbols* indicate cells encapsulated by alginate. The effects of encapsulation increase the concentration of extracellular protein

The use of encapsulating beads may be effectively combined with other strategies as discussed in earlier sections (inducible promoters, affinity bioreactors) to produce highly desirable results.

## 7 Conclusion

### 7.1 Summary

Clearly protein production in plant cell cultures is an exciting and promising frontier for new research. Plant cells have favorable growth characteristics and attributes (including the capacity for effecting complex modifications and protein secretion) that should help this technology compete effectively with other host systems. They can be genetically modified by well-established means and, in many cases, are highly stable. Plant cell cultures have demonstrated a capacity for producing a number of useful proteins both safely and effectively and potential for large-scale cultivation. Significant progress has been made in the last decade, but many challenges and unanswered questions remain. The key area for further research will be determining and overcoming the limiting factors in protein production to bring production levels of plant-produced recombinant proteins up to economically feasible levels. Protein production in plant cells is entering the most critical phase of its development, but there is significant cause for optimism. Future efforts must be based around novel approaches and innovative ideas that circumvent the root causes of the current production limits.

### 7.2
### Future Outlook

To some, the production of protein products in plant cell culture may appear as merely an odd curiosity: an interesting area for academic study, but showing little importance or promise for practical applications. More discerning minds may note that, over the development of the biotechnology industry, yeast expression systems found a place among bacterial systems and mammalian and baculoviral expression systems found a place among bacteria and yeast. Plant cell culture can produce functional, high-quality proteins at low to moderate expression levels. Considering the numerous advantages of plant cell culture over its closest competitors, including simplified purification, diverse processing options, and lack of human pathogens, the outlook for this technology is very bright.

## References

1. Tabata M, Fujita Y (1985) In: Zaitlin M, Day P, Hollaender A (eds) Biotechnology in plant sciences. Academic Press, San Diego, p 207
2. Remi Shih NJ, McDonald KA, Dandekar AM, Girbes T, Iglesias R, Jackman AP (1998) Plant Cell Rep 17:531
3. Lee J (1992) Biochemical engineering. Prentice Hall, Englewood Cliffs, NJ
4. An G (1985) Plant Physiol 79:568
5. Kusnadi AR, Nikolov ZL, Howard, JA (1997) Biotechnol Bioeng 56:473
6. Magnuson NS, Linzmaier PM, Gao J, Reeves R, An G, Lee JM (1996) Protein Exp Purif 7:220
7. Glick BR, Pasternak JJ (1994) Molecular biotechnology: principles and applications of recombinant DNA. ASM Press, Washington, DC
8. Liu F, Lee JM (1999) Biotech Bioproc Eng 4:259
9. Matsumoto S, Ikura K, Ueda M, Sasaki R (1995) Plant Mol Biol 27:1163
10. Magnuson NS, Linzmaier PM, Reeves R, An G, HayGlass K, Lee JM (1998) Protein Exp Purif 13:45
11. Wongsamuth R, Doran PM (1997) Biotechnol Bioeng 54:401
12. James E, Wang C, Wang Z, Reeves R, Shin JH, Magnuson NS, Lee JM (2000) Protein Exp Purif 19:131
13. Fischer R, Liao YC, Drossard J (1999) J Immunol Methods 226:1
14. Firek S, Draper J, Owen MRL, Gandecha A, Cockburn B, Whitelam GC (1993) Plant Mol Biol 23:861
15. Hogue RS, Lee JM, An G (1990) Enzyme Microb Technol 12:533
16. Tagge EP, Chandler J, Harris B, Czako M, Marton L, Willingham MC, Burbage C, Afrin L, Frankel AE (1996) Protein Exp Purif 8:109
17. Terashima M, Murai Y, Kawamura M, Nakanishi S, Stoltz T, Chen L, Drohan W, Rodriguez RL, Katoh S (1999) Appl Microbiol Biotechnol 52:516
18. Jones R, Robinson D (1989) New Phytologist 111:567
19. Lund P, Dunsmuir P (1992) Plant Mol Biol 18:47
20. Denecke J, Botterman J, Deblaere R (1990) The Plant Cell 2:51
21. Ma JKC, Hiatt A, Hein M, Vine ND, Wang F, Stabila P, van Dolleweerd C, Mostov K, Lehner T (1995) Science 268:716
22. Carpita N, Sabularse D, Montezinos D, Helmer DP (1979) Science 205:1144
23. Brodelius P, Nilsson K (1983) Eur J Appl Microbiol Biotechnol 17:275
24. Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS, Adams SP, Bittner ML, Brand LA, Fink CL, Fry JS (1983) Proc Natl Acad Sci USA 80:4803

25. Hiatt A, Cafferkey R, Bowdish K (1989) Nature (London) 342:76
26. Baez J, Russell D (2000) ACS National Meeting. San Francisco, California
27. Rodriguez RL, Lonnerdal B, Nandi S, Wu L, Huang J, Huang N (2000) ACS National Meeting. San Francisco, California
28. Nikolov Z, Kusnadi A, Woodard S, Azzoni A, Miranda E (2000) ACS National Meeting. San Francisco, California
29. Mason HS, Tacket CO, Richter LJ, Arntzen CJ (1998) Res Immunol 149:71
30. Fischer R, Drossard J, Commandeur U, Schillberg S, Emans N (1999) Biotechnol Appl Biochem 30:101
31. Conrad U, Fiedler U (1994) Plant Mol Biol 26:1023
32. Trudel J, Potvin C, Asselin A (1995) Plant Sci 106:55
33. Fischer R, Emans N, Schuster F, Hellwig S, Drossard J (1999) Biotechnol Appl Biochem 30:109
34. Sehnke PC, Pedrosa L, Paul AL, Frankel AE, Ferl RJ (1994) J Biol Chem 269:22473
35. Birch RG (1997) Annu Rev Plant Physiol Plant Mol Biol 48:297
36. Gao J, Lee JM, An G (1991) Plant Cell Rep 10:553
37. Hadi MZ, McMullen MD, Finer JJ (1996) Plant Cell Rep 15:500
38. Potrykus I (1990) Ciba Found Symp 1990 154:198
39. Rhodes CA, Pierce DA, Mettler IJ, Mascarenhas D, Detmer JJ (1988) Science 240:204
40. Laursen CM, Krzyzek RA, Flick CE, Anderson PC, Spencer TM (1994) Plant Mol Biol 24:51
41. Joersbo M, Okkels FT (1996) Plant Cell Rep 16:219
42. Wang AS, Evans RA, Altendorf PR, Hanten JA, Doyle MC, Rosichan JL (2000) Plant Cell Rep 19:654
43. Elliott AR, Campbell JA, Dugale B, Brettell RIS, Grof CPL (1999) Plant Cell Rep 18:707
44. Allen GC, Hall G Jr, Michalowski S, Newman W, Steven S, Weissinger AK, Thompson WF (1996) Plant Cell 8:899
45. Wahl MF, An G, Lee JM (1995) Biotech Lett 17:463
46. Ryland JR, Linzmaier PM, Lee JM (2000) J Microbiol Biotech 10:449
47. LaCount W, An G, Lee JM (1997) Biotech Lett 19:93
48. Sharp J, Doran PM (1999) Biotechnol Bioprocess Eng 4:253
49. Bateman KS, Congiu M, Tregear GW, Clarke AE, Anderson MA (1997) Biotechnol Bioeng 53:226
50. Chavan AJ, Haley BE, Volkin DB, Marfia KE, Verticelli AM, Bruner MW, Draper JP, Burke CJ, Middaugh CR (1994) Biochemistry 33:7193
51. Vuillard L, Madern D, Franzetti B, Rabilloud R (1995) Anal Biochem 230:290
52. Xie G, Timasheff SN (1997) Protein Sci 6:211
53. Schena M, Lloyd AM, Davis RW (1991) Proc Natl Acad Sci USA 88:10421
54. Van der Zaal EJ, Droog FNJ, Boot CJM, Hensgens LAM, Hoge JHC, Schilperoort RA, Libbenga KR (1991) Plant Mol Biol 16:983
55. Yoshida K, Kasai T, Garcia MRC, Sawada S, Shoji T, Shimizu S, Yamazaki K, Komeda Y, Shinmyo A (1995) Appl Microbiol Biotechnol. 44:466
56. Mett VL, Lochhead LP, Reynolds PHS (1993) Proc Natl Acad Sci USA 90:4567
57. Gatz C, Kaiser A, Wendenburg GR (1991) Mol Gen Genet 227:229
58. Claes B, Smalle J, Dekeyser R, Van Montagu M, Caplan A (1991) Plant J 1:15
59. Chan MT, Chao YC, Yu SM (1994) J Biol Chem 269:17635
60. Caddick MX, Greenland AJ, Jepson I, Krause KP, Qu N, Riddell KV, Salter MG, Schuch W, Sonnewald U, Tomsett AB (1998) Nat Biotechnol 16:177
61. Boetti H, Chevalier L, Denmat LA, Thomas D, Thomasset B (1999) Biotechnol Bioeng 64:1
62. James E (2001) Ph.D Thesis, Washington State University
63. Fischer R, Vaquero-Martin C, Sack M, Drossard J, Emans N, Commandeur U (1999) Biotechnol Appl Biochem 30:113
64. Gal S, Pisan B, Hohn T, Grimsley N, Hohn B (1992) Virology 187:525
65. Shen WH, Hohn B (1995) J Gen Virol 76:965
66. Scholthof HB, Scholthof KG, Jackson AO (1996) Annu Rev Phytopathol 34:299
67. Frazier GC, White WK, Dougall DK (1989) Biotechnol Bioeng 33:313

68. Linzmaier PM (1998) PhD thesis, Washington State University
69. Hooker BS, Lee JM, An G (1990) Biotechnol Bioeng 35:296
70. Li GQ, Shin JH, Lee JM (1995) Biotech Techniques 9:715
71. Curtis WR, Emery AH (1993) Biotechnol Bioeng 42:520
72. Bohme C, Schröder MB, Jung-Heiliger H, Lehmann J (1997) Appl Microbiol Biotechnol 48:149
73. Sahai O, Knuth M (1985) Biotechnol Prog 1:1
74. Schmidt AJ, Lee JM, An G (1989) Biotech Bioeng 33:1437
75. Nilsson K, Brodelius P, Mosbach K (1987) Methods Enzymol 135:222
76. Bodeutsch T, James E, Lee JM (1999) AIChE National Meeting. Dallas, Texas

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# Large-Scale Plant Micropropagation

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Plant micropropagation is an efficient method of propagating disease-free, genetically uniform and massive amounts of plants *in vitro*. The micropropagation from cells can be achieved by direct organogenesis from hairy roots or regeneration via somatic tissue. Once the availability of embryogenic cell and hairy root systems based on liquid media has been demonstrated, the scale-up of the whole process should be established by an economically feasible technology for their large-scale production in appropriate bioreactors. It is necessary to design a suitable bioreactor configuration that can provide adequate mixing and mass transfer while minimizing the intensity of shear stress and hydrodynamic pressure. Automatic selection of embryogenic calli and regenerated plantlets using an image analysis procedure should be associated with the system. Using the above systems, it will be possible to establish an advanced plant micropropagation system in which the plantlets can be propagated without soil under optimal conditions controlled in plant factory.

The aim of this review is to identify the problems related to large-scale plant micropropagation via somatic embryogenesis and hairy roots, and to summarize the most recent developments in bioreactor design. Emphasis is placed on micropropagation technology and computer-aided image analysis, including the successful results obtained in our laboratories.

**Keywords.** Plant cell culture, Micropropagation, Bioreactor design, Embryogenic callus, Hairy root, Image analysis, Plant factory



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## 1 Introduction

Plant micropropagation, an efficient method for propagating disease-free, genetically uniform plants in massive amounts *in vitro* is favored over propagation by conventional means such as cuttings and seeds because it offers various advantages [1]. The micropropagation from cells can be achieved by direct organogenesis via shoots or by somatic embryogenesis. Organogenesis via shoots is considered to be one of the most widely used commercial methods of regeneration. However, the procedures are labor-intensive and more specialized due to the many steps with manual manipulations involved and the low multiplication rates. Gradual acclimatization of plants to the greenhouse and then to the field is also needed. These numerous steps are accompanied by extensive costs and the commercialization has been limited to highly valuable crops [2]. The possibility for micropropagation through somatic embryogenesis provides a valuable alternative to the above propagation system [3–6]. This is amenable to a higher degree of automation and holds much promise for the mass propagation of plants at low cost because very large numbers of somatic embryos can be produced in a short period of time in a limited volume of medium. This should help towards making such a system economically viable for many plant species. The potential applications of somatic embryogenesis in plant breeding depend to a large extent on whether embryos develop through callus or directly from explant cells. The use of hairy roots has consistently been focused on the large-scale production of useful products or secondary metabolites [7–10]. However, the micropropagation system sheds light on other potentials for the agronomic application of hairy roots. To date, plant regeneration from hairy roots has been reported in several species and some characteristics of hairy roots, such as the stable regeneration ability, have implications for the development of plant regeneration systems [11–14]. In addition, the use of *Agrobacterium rhizogenes* offers the opportunity to introduce foreign genes into plant genomes when a hairy root is reduced, enabling the alteration of plant's properties by gene manipulation [15]. Thus, the micropropagation of elite hairy roots offers other attractive advantages in the large-scale production of artificial seeds.

Once the availability of embryogenic cell and hairy root systems based on liquid media has been demonstrated, the systems makes it possible to produce somatic embryos and hairy roots in large systems and to scale-up the whole process to an economically viable technology. Large-scale production of somatic embryos and hairy roots in appropriate bioreactors is essential if micropropagation and artificial seed systems are to compete with natural seeds. It is known that plant cells are relatively large in size and have relatively low resistance to hydrodynamic or shear stress. When cells in suspension are subjected to moderate levels of hydrodynamic or shear stress, they will tend to deform or rupture, causing cell death [16]. Therefore, it is necessary to design a suitable bioreactor configuration which may be operated at a large scale that can provide adequate mixing and mass transfer while minimizing the intensity of shear stress and hydrodynamic pressure. Immobilization technology as applied wid-

ely in the production of secondary metabolites by plant cells has been developed successfully [17–19]. Immobilization not only promotes cell-to-cell contact which can enhance the accumulation of secondary metabolites [20], but also protects the plant cells from hydrodynamic and shear forces, thus improving the production of embryogenic callus with a high regeneration potency [21, 22]. Combined with the rapid progression of bioreactor technology, some novel immobilized cell systems are being developed for the scaling-up of the production process.

For the mass production of regenerated plantlets, embryogenic calli should be selected. Manual screening by human experts is labor-intensive, time-consuming, and expensive. Therefore, some automatic selection systems using image analysis should be developed. Most embryo cultures give rise to a heterogeneous population including embryos at various stages of development, abnormal embryos, and callus. This population heterogeneity necessitates a sorting step to select for normal and mature embryos [23]. Further optimization of bioreactor operation is often hampered by the difficulty in evaluating the results. Typically, an operator examines a population sampled from a culture microscopically and classifies the embryos into developmental classes and enumerates them. The process is subjective and tedious, and often results in a low degree of statistical confidence due the fact that often only a small number of embryos is classified and counted. An advanced image analysis and pattern recognition system is therefore needed to discern the development of the somatic embryos automatically in the process control and optimization. In addition, plantlets obtained via regeneration in a bioreactor need to be transferred onto a solid medium and acclimatized under light irradiation in order to obtain healthy plants. In this step, the transfer time is different for each plantlet, only plantlets with a long shoot, which confers high photosynthesis ability, should be transferred. An automatic selection system using image analysis is also desired for this purpose.

The aim of this review is to identify the problems related to large-scale plant micropropagation via somatic embryogenesis and hairy roots, and to summarize the most recent developments in bioreactor design. Emphasis is placed on immobilization technology and computer-aided image analysis employed in the mass micropropagation, including the successful results obtained so far.

## 2 Bioreactor Design for Large-Scale Micropropagation

The design and operation of a bioreactor are mainly determined by biological needs and engineering requirements, which often include a number of factors: efficient oxygen transfer and mixing, low shear and hydrodynamic forces, effective control of physico-chemical environment, easy scale-up, and so on. Because some of these factors can be mutually contradictory, it is difficult to directly employ a conventional microbial reactor to shear-sensitive plant tissue cultures.

Different reactor configurations for plant cells, tissue and organ cultures can be found in previous publications by Prenosil and Pederson [24], Scragg and

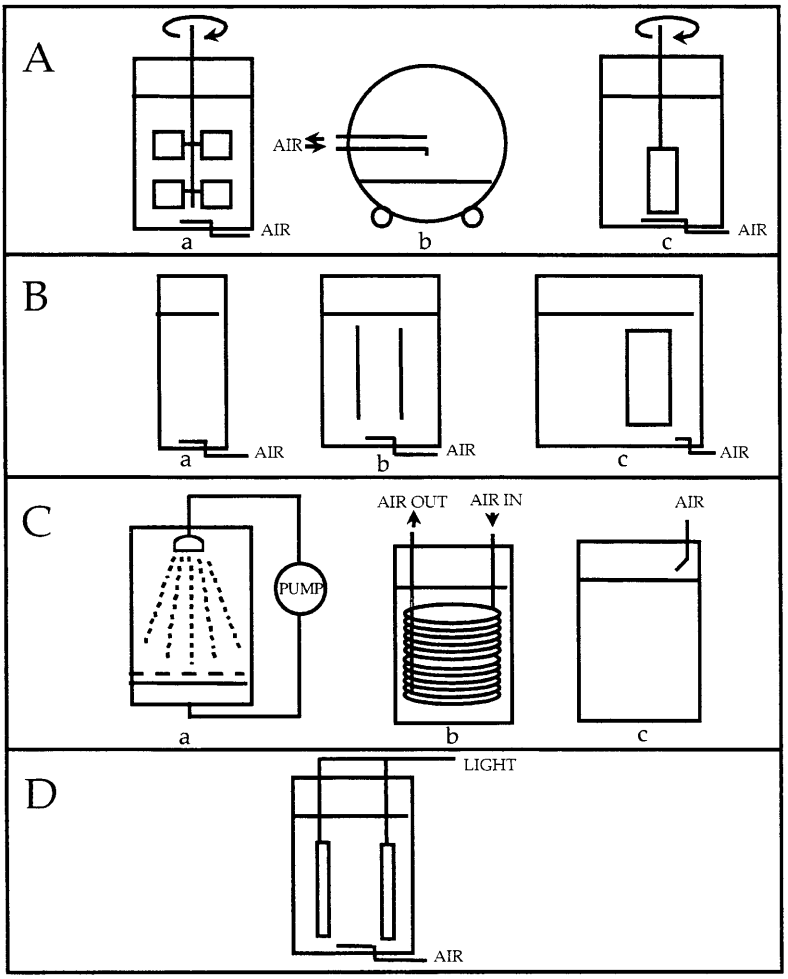


**Fig. 1.** Different types of bioreactors for plant cell, tissue and organs. (*A*) mechanically-agitated bioreactors, a; aeration-agitation, b; rotating drum, c; spin filter. (*B*) air-driven bioreactors, a; bubble column, b; draft tube, c; external loop, (*C*) non-agitated bioreactors, a; gaseous phase (mist), b; oxygen permeable membrane aerator, c; surface aeration, (*D*) light emitting draft tube

Flower [25], Panda et al. [26], Doran [27]. and Payne et al. [28]. Several kinds of bioreactors, such as the stirred tank bioreactor with hollow paddle and flat blade impellers, the bubble column, the airlift bioreactor with internal and external loops, the rotating drum bioreactor, the stirred-tank with a draft tube, and the mist bioreactor have been attempted for plant cell, tissue and organ cultures (Fig. 1).

## 2.1
## Plant Suspension Cells

Among the bioreactors available for cultivation of shear-sensitive plant cells, some modified conventional stirring tanks, which are effective in the mixing of the contents, the suspension of cells, and the break-up of air bubbles for improved aeration have been developed by employing a variety of impeller designs [29–32]. Consequently, these bioreactors have great potential when used at a mild agitation intensity and have received increasing attention.

A recently developed bioreactor with a helical-ribbon impeller has been effectively used in high-density plant cell culture (Fig. 2). This bioreactor displayed good performance for growing high-density *Catharanthus roseus* suspension cultures with high cell viability and embryogenic cultures of a transformed *Eschscholtzia californica* cell line [33, 34]. In the bioreactor, a larger double helical-ribbon impeller with the baffles exhibited a uniform low-shear mixing of cultures with a sufficient supply of oxygen without excessive foaming and biomass flotation.

Recently, Wang and Zhong successfully designed a novel centrifugation impeller bioreactor (Fig. 3) for shear-sensitive cell systems [35, 36]. They proved

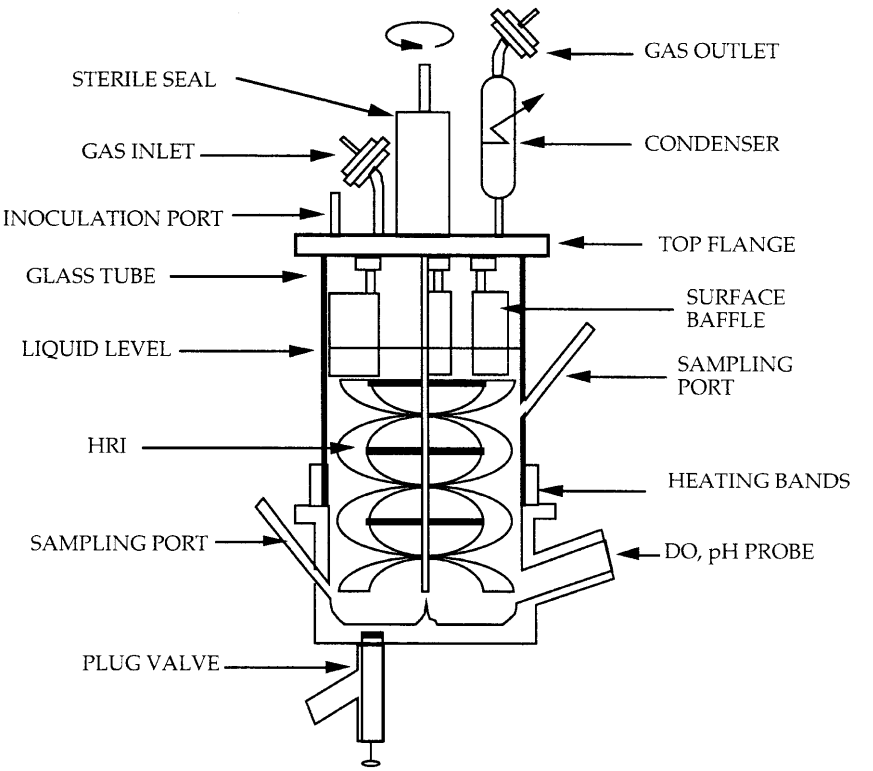


**Fig. 2.** Configuration of helical ribbon type bioreactor (HRI)

that the new bioreactor possessed several advantages over a widely used cell-lift reactor. These include much higher liquid lift capacity, better mixing performance, lower shear stress and surface liquid turbulence, which can cause a serious loss of cell viability. In addition, when a sintered stainless sparger with tiny pore size was installed about 4 cm off the center with respect to the draft tube of the impeller, a high value of oxygen transfer was obtained under low hydrodynamic forces.

Considering bioreactor operation, such as medium exchange, a spin-filter bioreactor has been recognized as one of the most suitable designs for mass propagation via somatic embryogenesis [37, 38]. In the bioreactor, the spent medium was removed without cell washout through the spinning central filter, which causes agitation of the medium without generating shear forces. In the meantime, fresh culture medium that stimulates embryo differentiation is supplied into the bioreactor. In order to realize continuous callus cell proliferation and embryo development on a large scale, two spin-filter bioreactors were configured in series, as shown in Fig. 4. A more efficient strategy would be operation of the first-stage bioreactor (cell proliferation) as a continuous culture. Continuous culture provides more homogeneous and constant culture conditions for the cells and maintains an actively-growing cell population available

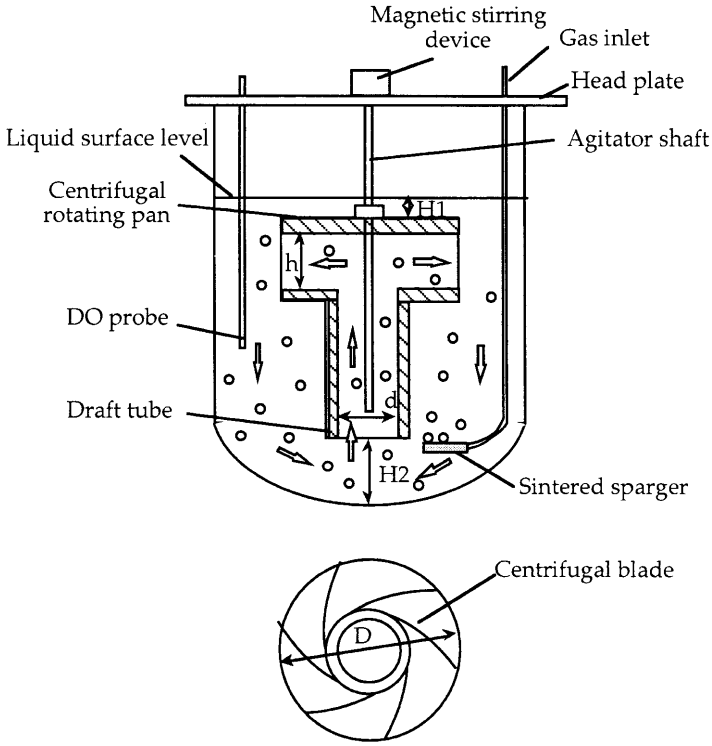


**Fig. 3.** Schematic diagram of centrifugal impeller bioreactor (5 L)

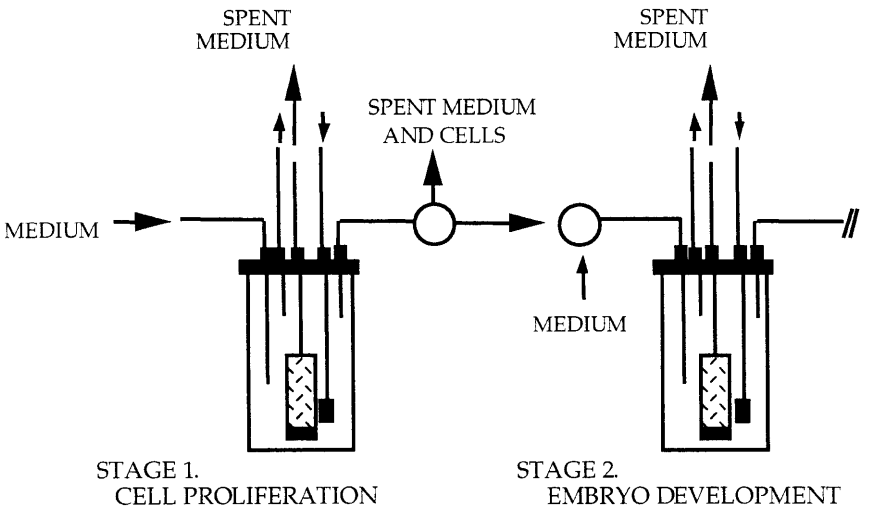


**Fig. 4.** Two spin-filter bioreactors configured in series for the growth of embryogenic cells and the promotion of somatic embryo maturation on a large scale. The stage 1 bioreactor is for cell proliferation and operates on a continuous culture mode; the stage 2 bioreactor is for somatic embryo maturation and operates in a perfusion mode

for embryo development at all times. The second-stage bioreactor results in the periodic production of embryos. The embryo development could be controlled by way of nutrient requirements and air components. Carrot embryogenic suspensions and mature somatic embryos have been successfully grown using spin-filter bioreactors on a large scale. When moved to a growth medium, the embryos produced in the bioreactor developed into normal plants.

### 2.2 Hairy Roots

Owing to the unique configuration of hairy roots with their branching nature, some specific engineering aspects of bioreactor design should be considered, including a support matrix to support the roots, protection from shear stress, homogenous growth distribution, and oxygen transfer enhancement in the interwoven root matrix [39–43].

We reported that an airlift column reactor was superior for the cultivation of hairy roots, in which reticulate polyurethane foam was used as an appropriate support for the even growth and distribution of the hairy roots [44]. A turbine-blade reactor was developed by Nagai et al. [45], in which the cultivation space is separated from the agitation space by a cylindrical stainless-steel mesh and a stainless-steel plate with a slit so that hairy roots do not come into contact with the impeller. The impeller, with 8 turbine blades, is fitted in the agitation space at the bottom of the reactor. The medium flows upwards along the vessel wall, passes through the cylindrical stainless-steel mesh in the center of the re-

actor, and then returns to the agitation space. The bioreactor was found to be advantageous for hairy root culture because of a high oxygen transfer [46]. Furthermore, a high density of final biomass was obtained in the bioreactor by fed-batch culture [47].

Murashige [48] has proposed the concept of "artificial seed" as an approach to the mass propagation of elite plant varieties. Artificial seeds are expected to be a reliable delivery system for the clonal propagation of elite plants [49–51]. The delivery system has the potential for genetic uniformity, high yield, and low cost of production. Hairy root methodology is one of the promising candidates as material for artificial seed.

We focused on the development of a micropropagation procedure for hairy roots by using an artificial seed system from the standpoint of bioengineering [52]. Horseradish hairy roots generated by the integration of a part of the Ri plasmid in *Agrobacterium rhizogenes* were excised and encapsulated in alginate beads to produce individual artificial seeds [53]. Root fragments with an apical meristem of a branch efficiently regenerated to the whole plants, and those more than 5 mm in length possessed a high shoot-formation ability. We found that naphthaleneacetic acid (NAA) or indole-3-butyric acid treatment stimulated branch emergence and shoot formation on the roots. In order to stimulate plantlet formation from the hairy roots, the effects of kinetin supplementation at the plantlet formation stage on plantlet formation were also tested [54, 55].

Fig. 5 shows an artificial seed production system using plantlets derived from hairy roots. The hairy roots must be cut to produce the artificial seeds. Fragmentation was carried out using a commercial blender with blades. Horseradish hairy roots cultured for 35 days without addition of plant growth

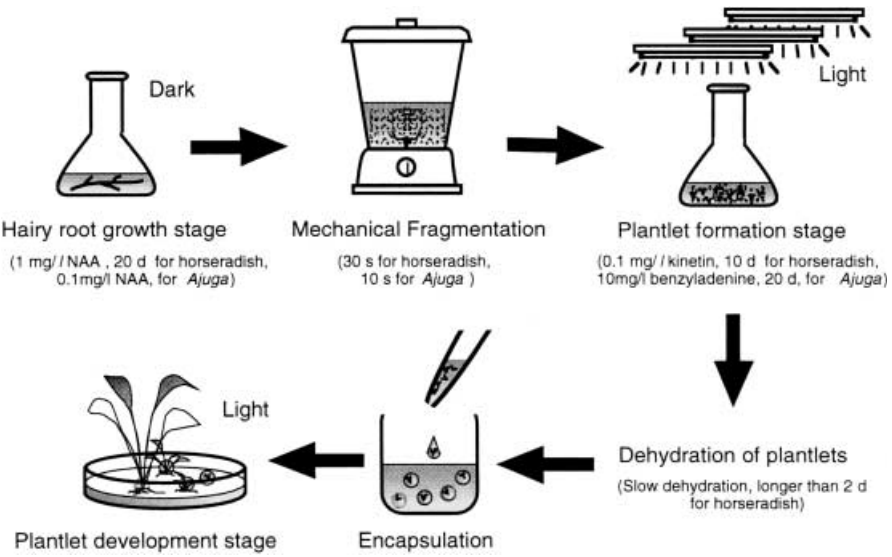


**Fig. 5.** Artificial seed production system using plantlets derived from hairy roots

regulator were fragmented in the blender for 10, 20, 30, or 60 seconds. Approximately 54% of the root fragments (ca. 100 mm) on a fresh weight basis was recovered in the case of fragmentation for 30 s. After fragmentation, the root fragments were transferred into plantlet-formation medium without plant growth regulator. Shoots appeared at day 4, and secondary roots began to elongate from the root fragments. The highest plantlet formation frequency was found to be achieved in the medium supplemented with 0.1 mg/L of kinetin. The effect of plantlet dehydration on the frequency of plantlet development was also examined. After 7 days of dehydration treatment in a sealed Petri dish, 98% of the encapsulated plantlets had developed into healthy plants at 15 days of culture [56, 57]. The proposed system was applied efficiently to the micropropagation of transgenic *Ajuga* hairy roots [58].

The fragments of hairy roots can be incubated under illumination for regeneration of plantlets. In view of this process, a shaking flask vessel-type bioreactor (Fig. 6), which is not associated with an impeller for mixing and itself is shaken in a rotational mode, has been successfully used for large-scale plantlet propagation from the fragmented pieces of horseradish hairy roots. A lower shear rate is generally exerted on the cultures as compared with a conventionally agitated vessel with an impeller, and the oxygen requirement by plant cells is supplied from the free surface of the medium so that the cultures are never exposed to a hydrodynamic stress by bubbling. Therefore, almost the same

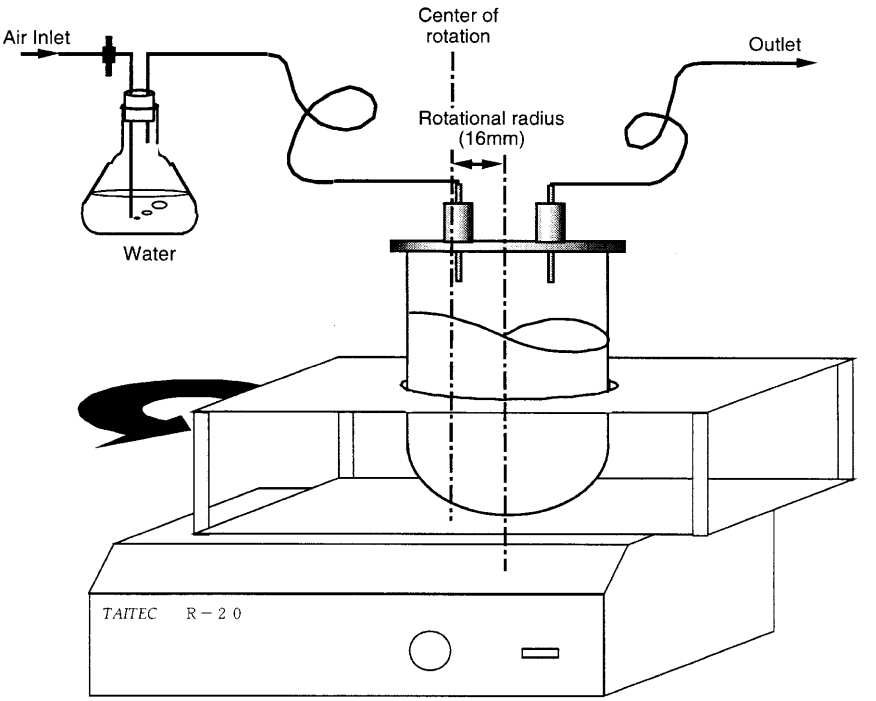


**Fig. 6.** Schematic diagram of a shaking-vessel type bioreactor

number of regenerated plants at a rotational speed of 120 rpm was obtained compared with that in the control culture using a flask. Furthermore, the possibility to scale up the bioreactor and operate it successfully has been proved [59].

## 3
## Immobilization Technology in Large-Scale Micropropagation

### 3.1
### Plantlet Regeneration from Immobilized Calli in Gel Beads

Immobilization is an important strategy for the removal of shear stress. Gel entrapment culture using calcium alginate gel has been reported by many researchers [60–62]. We also investigated the immobilized gel beads culture of embryogenic celery callus [21]. Our advanced idea was that celery embryos and plantlets were released in a culture of immobilized Ca-alginate gel beads in which celery callus was entrapped under regeneration conditions. The culture procedure and a schematic diagram of embryo and plantlet regeneration from the immobilized callus are shown in Fig. 7. The cells released from the gel bead were larger than those obtained in suspension culture. The optimal concentration of alginate gel for embryo and plantlet production was 2% for the immobilized cell culture. Considering the maintenance of the gel bead structure and the detrimental effect of $CaCl_2$ on plantlet development, 5 mM $CaCl_2$ supplementation gave the best result in terms of the number of heart and torpedo embryos and plantlets. The ratio of the number of heart embryos, torpedo embryos, and plantlets to the total number of cells in the immobilized cell culture was higher than that in the suspension culture. Repeated batch culture with 5 mM $CaCl_2$ provided long-term (more than 154 days) embryo and plantlet production without disruption of the gel beads. The productivity of plantlets in the immobilized cell culture with 5 mM $CaCl_2$ was 2.2-fold higher than that in the suspension (Table 1). The attractive immobilization release system was also used in the regeneration of carrot cells [22]; repeated batch culture for plantlet

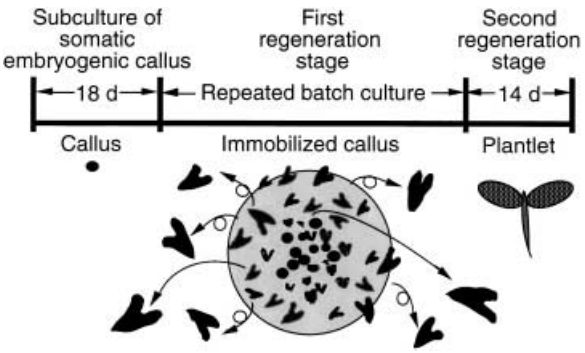


**Fig. 7.** Culture procedure and schematic diagram of embryo and plantlet regeneration from the immobilized callus

**Table 1.** Total number of embryos and plantlets in the suspension culture and in the immobilized culture

| | Suspension (1/L) (42 d)[b] | Immobilization with $CaCl_2$ (1/L)[a] | | |
|---|---|---|---|---|
| | | 0 mM (126 d)[b] | 5 mM (154 d)[b] | 10 mM (154 d)[b] |
| Heart and torpedo embryos + plantlets[c] | $6.1 \times 10^4$ | $1.7 \times 10^5$ | $2.1 \times 10^5$ | $1.0 \times 10^5$ |
| Plantlets[d] | $2.1 \times 10^4$ | $1.4 \times 10^5$ | $1.7 \times 10^5$ | $7.2 \times 10^4$ |
| Plantlets per day[e] | $5.0 \times 10^2$ | $11 \times 10^3$ | $1 \times 10^3$ | $4.7 \times 10^2$ |

[a] All of the repeated batch cultures were carried out in the medium of the first regeneration stage containing 0,5 or 10 mM $CaCl_2$.
[b] The duration of the culture is shown in parentheses.
[c] Total number of heart and torpedo embryos and plantlets in the medium. The heart and torpedo embryos and plantlets were counted at the end of the first regeneration stage.
[d] Total number of plantlets at the end of the second regeneration stage. The cells obtained from the first regeneration culture were grown in the second regeneration culture. The number of plantlets was evaluated at the end of the second regeneration stage.
[e] The number of plantlets produced per day.

production continued for 245 days with no significant decrease in the productivity ($1.6 \times 10^5$ plantlets/L-medium/day) (Table 2).

In the above immobilized gel beads system, the cells were entrapped in alginate gel and protected from physical stress. The released cells were immediately recovered from the culture. Thus, the immobilized gel beads system was expected to be easily scaled up because the production of heart embryo, torpedo embryos, and plantlets could be improved by increasing the number of gel beads in a bioreactor. A rotating-mesh basket type bioreactor with a cyclone type cell separator (Fig. 8) was designed and used in the immobilized gel beads system for the embryo production and continuous recovery of released cells [63]. Plantlet formation of the released cells from the rotating mesh-basket type reactor culture reached a level of 115% compared with that from the flask culture. When 110 mL/min of medium was allowed to flow through the separator, 90% of the suspension cells were collected after 40 min of operation time. At the rotation speed of 100 rpm, constant values of released cell volume of 3 mL-pcv/L-medium/day and plantlet numbers of about 7000 plantlets/g released cells were obtained over 12 days (Fig. 9). The volumetric productivity was 21,000 plantlets/L medium/day, which corresponds to the number of plantlets able to be sowed in a 0.1 ha field. This bioreactor system has great potential for the mass-production of regenerated plantlets. Furthermore, large numbers of plantlets will be obtainable because the size of the mesh-basket and packed beads volume can be increased easily. When 0.4 L (3700 beads) beads per L-mesh-basket were packed in the bioreactor, the ability to form plantlets was similar to that in the experiment with 0.1 L beads per L-mesh-basket and the total number of plantlets was about 4-fold higher. From these results, the bioreactor system proposed by us was concluded to be applicable for plantlet production as a strong tool for micropropagation in plant factories.

**Table 2.** Summary of long-term repeated batch culture using immobilized gel beads

| | Immobilized culture supplemented with $CaCl_2$ | | | | | Suspension culture |
|---|---|---|---|---|---|---|
| | 0 mM | 5 mM | 10 mM | 15 mM | 20 mM | |
| Average of value in one batch operation | | | | | | |
| Relative value of released cell volume [a] (–) | 0.8 | 0.8 | 0.7 | 0.5 | 0.3 | 1[b] |
| Relative value of regeneration ability [a] (–) | 1.5 | 1.4 | 1.2 | 1.0 | 0.9 | 1[c] |
| Number of plantlets produced (plantlets/*L*) | $2.1 \times 10^6$ | $2.0 \times 10^6$ | $1.4 \times 10^6$ | $0.93 \times 10^6$ | $0.58 \times 10^6$ | $1.8 \times 10^6$ |
| Cultivation time (d) | 161 | 245 | 259 | 161 | 161 | |
| Number of batch operations | 13 | 19 | 20 | 13 | 13 | |
| Cumulative number of plantlets (plantlets/L-medium) | $2.9 \times 10^7$ | $4.0 \times 10^7$ | $2.9 \times 10^7$ | $1.3 \times 10^7$ | $0.8 \times 10^7$ | |
| Productivity (plantlets/*L*/d) | $1.8 \times 10^5$ | $1.6 \times 10^5$ | $1.1 \times 10^5$ | $0.81 \times 10^5$ | $0.50 \times 10^5$ | $1.3 \times 10^5$ |

[a] Relative values were calculated with respect to the value in the suspension culture. Regeneration ability was determined with an inoculum size of 0.2 mL-pcv in the regeneration medium.
[b] The released cell volume was 153 mL-pcv/L-medium.
[c] The number of regenerated plantlets was 12,000 plantlets/mL-pcv.

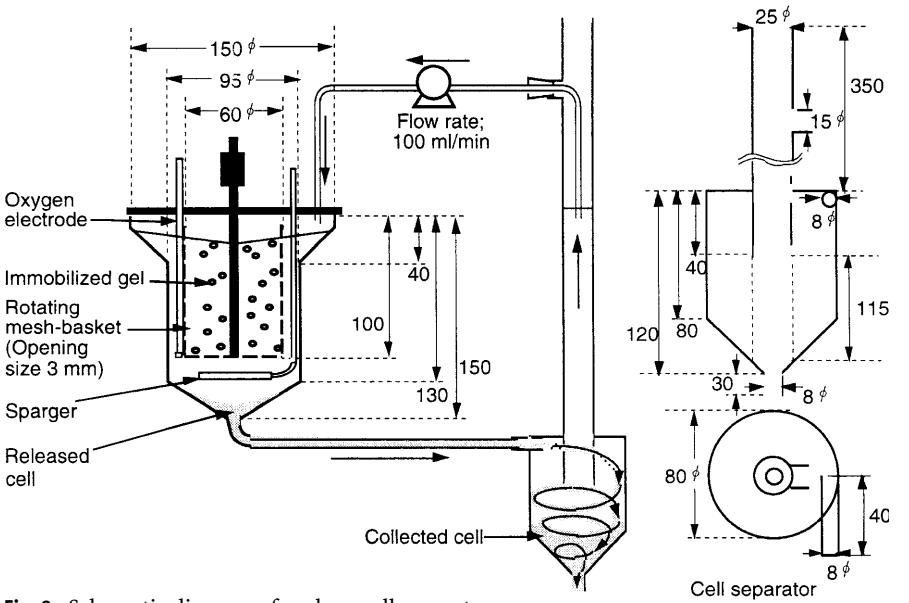


**Fig. 8.** Schematic diagram of cyclone cell separator

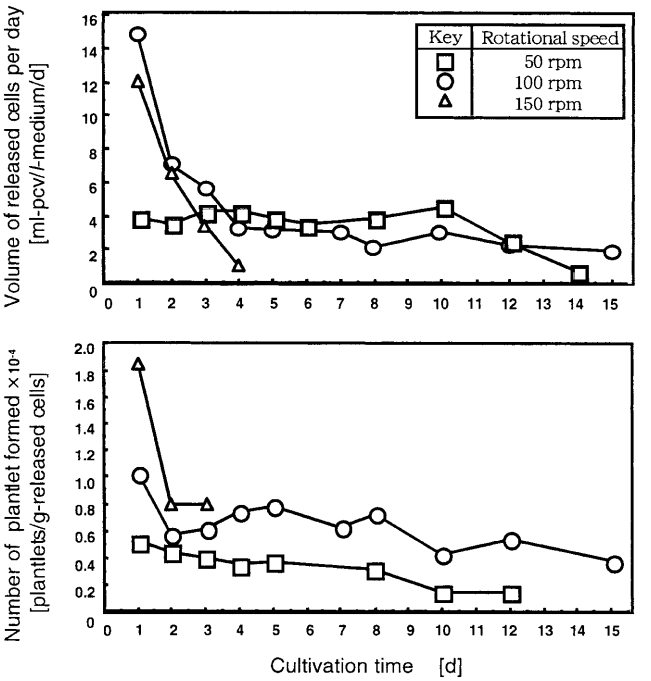


**Fig. 9.** Time courses of a long term culture in immobilized gel beads system. Number of plantlets formed at 4 d and 150 rpm and that 14 d of 50 rpm were not measured since the volume of released cells was too small

### 3.2
### Plantlet Regeneration from Immobilized Calli in Polyurethane Foam

Embryogenic rice calli tend to form larger clumps during cultivation. Therefore, immobilization of the calli has hardly been carried out until now. Porous supports such as polyurethane foam have often been used for the immobilization of mycelial cells [64, 65] and plant cells [66–68]. In almost all cases, effective production of biological materials by the immobilized cells has been reported. To avoid the damage due to the hydrodynamic stress, we proposed the immobilization culture of rice callus using a macroporous urethane foam support. A turbine-blade reactor (TBR), which has been developed for hairy root culture, was also used in the culture. In the culture space, polyurethane foam was added as an immobilization support.

Embryogenic rice calli were induced from mature rice (*Oryza sativa* L., Sasanishiki) seeded in N6 medium according to the previous paper [69]. Callus induction was carried out at 30 °C in the dark for 4 weeks in an incubator.

When 3-mm cubes of polyurethane foam with a pore size of 1.3 mm were used for immobilization, rice calli proliferated well and a total cell concentration of 6.8 g/L was obtained after 1 week of culture [70]. Rice calli were cultured in the TBR under various agitation speeds (200, 300, and 400 rpm) using the cube supports corresponding to 5 % by volume of 600 mL growth medium. The results are shown in Fig. 10 (triangles and broken lines). When the supports were used in the TBR, cell growth was not inhibited and there was no decrease in the regeneration frequency at high agitation speeds (300, 400 rpm), whereas cell growth inhibition was observed without supports. Support volume had a pronounced effect on immobilization frequency of rice callus. Maximum immobilization frequency was found at 60 mL. On 3-fold repetition of the periodic operation (agitating at 300 rpm for 5 min and then 50 rpm for 2 min, and then 200 rpm of constant agitation speed during the remaining time), the distribution of rice calli within the support became homogenous and their immobilization frequency was improved as compared with that using the constant bioreactor operation at 200 rpm. However, the immobilization frequency decreased due to an insufficient amount of the support for immobilization and the floating of the support by air flow. After the turbine blade bioreactor was modified by setting the air sparger at the bottom of cylindrical stainless mesh in order to reduce the floating of the support by the air flow, the immobilization frequency increased further and reached 86.3 % when we increased the support volume to 90 mL in the modified turbine blade bioreactor. The regeneration frequency of the immobilized callus was not affected by the support volume increase, periodic operation, and bioreactor modification when they were transferred on solid regeneration medium.

We developed an immobilized cell system for cultivation of embryogenic rice callus using polyurethane foam, and found that the immobilized callus maintained a high regeneration ability because shear stress and hydrodynamic damage were avoided [70, 71]. In these studies, however, regeneration was carried out on the solid medium. The clumps of calli grew larger on the solid medium for regeneration and larger clumps were more fragile. Moreover, the ratios of

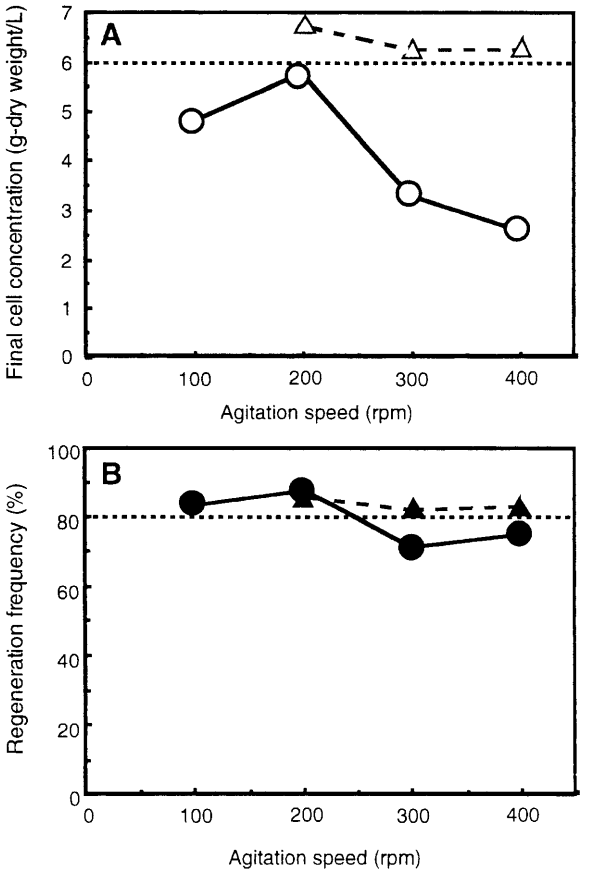


**Fig. 10.** Effect of agitation speed on cell concentration (**A**) and regeneration frequency (**B**) of rice calli in TBR without (○, ●) and with (△, ▲) supports. *Dotted lines* show the results of the control culture

the clumps with root and shoot were about 80%, not 100%. Therefore, it will be necessary to establish some sophisticated systems for handling of the regenerated callus when these calli were finally transplanted to the paddy soil in practical use. From these considerations, we hit upon the idea of direct plantlet regeneration of rice calli immobilized in polyurethane foam in liquid medium. This way will be convenient because our subjects are the calli immobilized in foam, not fragile clumps, and the foams exit in the liquid medium and are easy to transport.

On the basis of these results, the following novel regeneration system is proposed [73]. Immobilization supports are transferred directly into the regeneration medium and the regenerated calli are obtained *in situ* in the supports. Regenerated calli in the supports with 3 to 5 germinated shoots are incubated in a preculture nursery and then transferred to a normal paddy. This system,

that is *in situ* regeneration, is attractive since it is easily applicable to conventional agricultural procedures.

An *in situ* regeneration system is composed of the callus growth stage and the regeneration stage [73]. In the callus growth stage, 3-mm cubes of with pore size of 1.3 mm were employed. In the *in situ* regeneration stage, regeneration was carried out in liquid regeneration medium and a macroporous support suitable for immobilization in regeneration stage was investigated. Since it has been reported that the medium exchange regeneration culture was effective for *in situ* regeneration, the regeneration stage was divided into two periods. A schematic diagram of the *in situ* regeneration is shown in Fig. 11. The following two media were used [72]: A1 medium – 1/2 N6 medium (pH 5.8) supplemented with 30 g/L sorbitol, 10 g/L sucrose, 1.4 g/L proline, 2.0 g/L casamino acids, 0.4 mg/L NAA, 0.5 mg/L kinetin; A2 medium – 1/2 N6 medium (pH 5.8) supplemented with 15 g/L sorbitol, 7.5 g/L sucrose, 1.0 g/L casamino acids, 1.0 mg/L NAA, 0.5 mg/L kinetin. The rice calli of 7-day culture using N6 medium, which are mainly 1–2 mm of clump size, were transferred to A1 liquid medium for the first stage of regeneration. After 20 days, the medium was changed to A2 medium and the liquid culture was continued as the second stage of regeneration in this experiment. In the first stage, the embryogenic calli began to differentiate, and the callus size ranged mainly from 1.0 mm to 2.8 mm. No shooting plantlets were observed in the first stage of regeneration although some green spots were observed. On the contrary, in the second stage, the callus size increased quickly. Rice calli between 2.8 to 4.0 mm and above 4.0 mm in diameter accounted for 48.1% and 31.4% of the mass, respectively, after 40 days. Most of them were regenerated plantlets with shoots. From these findings, the callus immobilization was conducted in the second stage after the medium exchange. From the size distribution, we prepared three kinds of polyurethane foam with larger pore sizes, 1.9, 3.6, and 5.1 mm. A 10-mm support cube with pore size of 3.6 mm gave the most highly efficient immobilization and *in situ* regeneration of rice callus. Rice calli after 7-day culture using N6 medium were transferred to A1 medium, and cultured for a given number of days. At that time, the medium was changed to A2 medium with the addition of the support cubes, and the calli were cultured for the rest time of the 40-day period. The optimum duration of the first stage in A1 medium was 15 days and the percent of support cubes with 3–5 plantlets reached 61.0% after 40 days. Compared with the result at the optimum time of medium exchange, the ratio of *in situ* regeneration decreased in other cases. This may be due to the fact that the amount of nutrients and the osmotic pressure in the medium should be regulated according to the different development stages of the regeneration phase [72], or to some inhibitory factors that might be released from the embryogenic calli that were removed [74]. When the rice callus was cultivated with support cubes in 60 mL of A2 medium in a 500-mL flask, 83% of the immobilization ratio was accomplished and 82% of support cubes contained 3–5 regenerated plantlets after 25 days. The percent of support cube with 3–5 plantlets was limited at a lower exchanged medium volume of 30 mL. This may be due to the lack of nutrients. However, at a higher exchanged medium volume of 90 mL, the support cubes were submerged completely in liquid medium during the regeneration period,

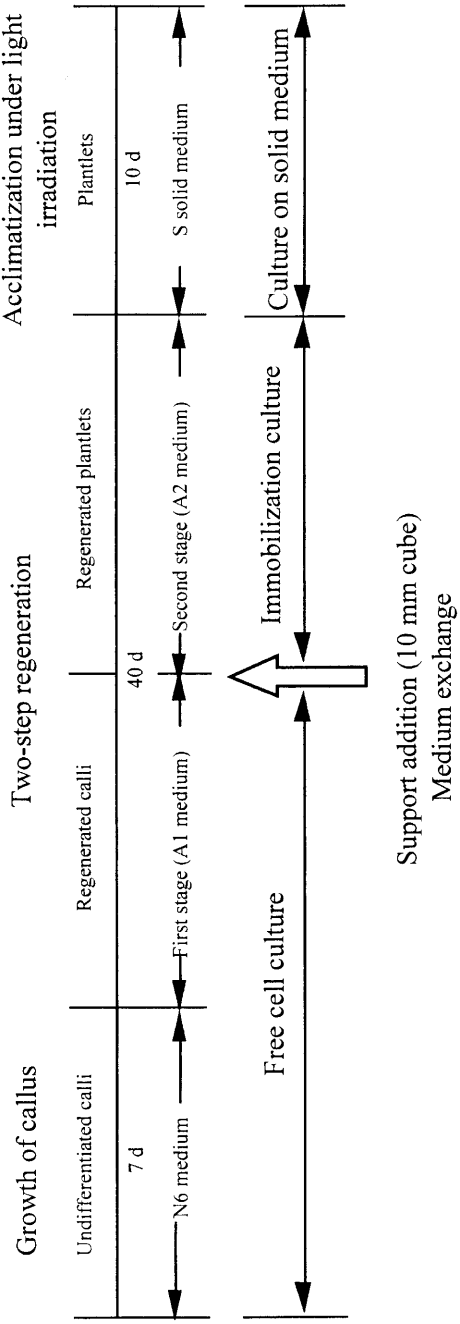


**Fig. 11.** Schematic diagram of *in situ* regeneration culture of rice callus

and the plantlet development was also inhibited. This might be related to the limitation of oxygen transfer to the immobilized calli in the support cube. Shoot length of regenerated plantlets obtained from the *in situ* regeneration culture was longer than that from the suspension culture. In this experiment, the support cubes with 3–5 regenerated plantlets from the 500-mL flask were transferred to 1/4 MS solid medium supplemented with 10 g/L sorbitol and 5 g/L sucrose (S medium). After 10 days, 3–5 regenerated plantlets developed quickly into 3–5 plants with a length above 10 cm (Fig. 12). These healthy plants were easy to transfer to photosynthetic growth in low nutrient broth. This means that the plants could be transferred to a normal paddy in the practical agricultural procedures after further acclimatization.

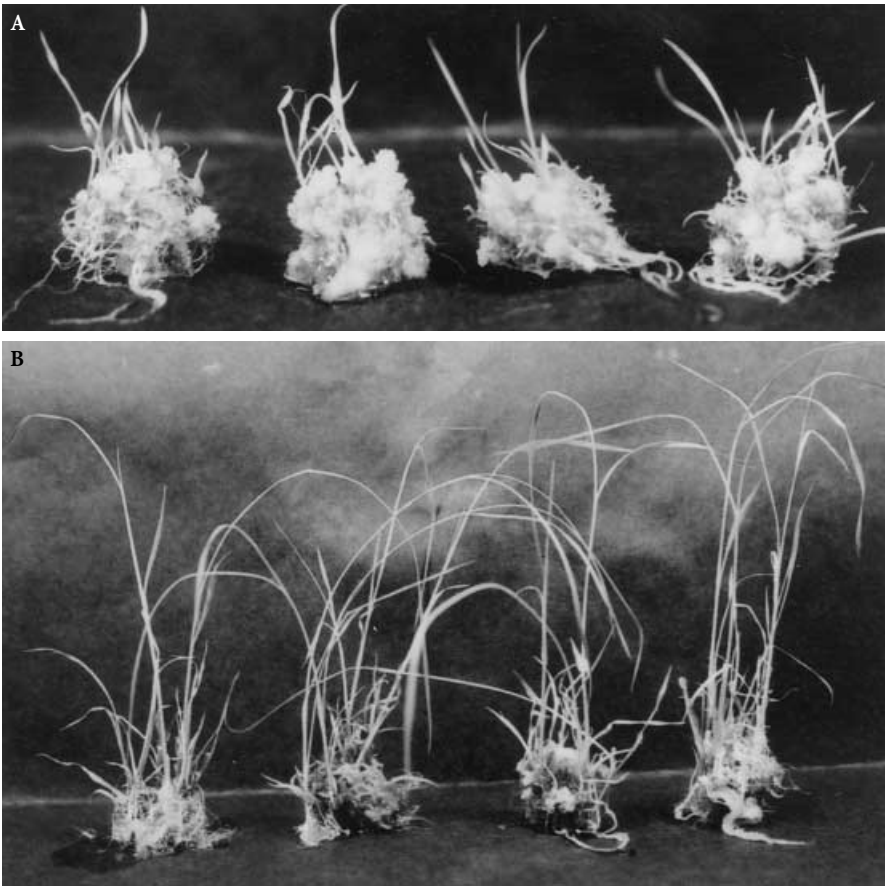


**Fig. 12.** Photograph of regenerated rice calli (*A*) and developed plantlets (*B*) in 10-mm support cube with the pore size of 3.6 mm

# 4
# Image Analysis System for Large-Scale Micropropagation

Process engineering including bioreactor design, high cell density culture, image analysis with computer device, and robotic system can improve the micropropagation process. To establish the process for plant micropropagation, some problems are still open, except for bioreactor design. The following three subjects should be overcome by image analysis. One is the selection of embryogenic callus. Many researchers have reported the induction and propagation of somaclonal callus from various species of plants. Such callus clones, however, do not all exhibit embryogenicity. A certain percentage of embryogenic calli cannot generate shoots and roots and never regenerate. A second one is the selection of embryos. Embryogenic callus is never homogeneous, and contains various calli, which are in the different stages of regeneration or development. In general, somaclonal embryos can be classified in three stage for development, globular, heart, and torpedo type embryos. A third point is the decision of the timing of transferring to an acclimatization medium. Regenerated plantlets with a sufficient length of shoot can be transferred to the low nutrient medium for photosynthetic growth.

## 4.1
## Selection of Embryogenic Callus

In the cultivation of plant calli on solid media, two kinds of calli the so-called compact and friable calli, which are a bright yellow and a whitish clump, respectively, are often obtained. The distinction of these calli is of great importance in the regeneration step.

In our work [75], 8 clones of sugarcane callus from three subtypes, 4 compact callus clones and 4 friable ones, were used. For automatic selection of calli, the image analysis system constructed by us was used, which consisted of a microscope, a Charge Coupled Device (CCD) camera, and a ring type light source. Calli in sterilized dishes were used *in situ* for image analysis. The original image of the callus was input to a computer via an image analysis board. Using Adobe Photoshop 3.0 J software, only the image of the region of the callus was extracted.

The brightness of yellow, $Br(Y)$, and white, $Br(W)$, were defined using $Br(R)$, $Br(G)$, and $Br(B)$. These were defined as follows.

$$Br(Y) = \text{Minimum}\,[Br(R), Br(G)]$$

$$Br(W) = \text{Minimum}\,[Br(R), Br(G), Br(B)]$$

Here, Minimum [A, B, C] is a function to determine the smallest value among A, B, and C. The difference between $Br(Y)$ and $Br(W)$, $Br(Y–W)$, which can be used to express the yellowish grade, was calculated.

When $Br(Y–W)$ was determined from all pixels of the original images of both calli, the compact calli were found to be clearly distinguished from the friable calli by the frequency distributions of $Br(Y–W)$. The typical frequency

distributions of $Br(Y-W)$ for compact and friable calli are shown in Fig. 13. The average brightness center values of the 20 compact and friable callus images were 32.3 and 11.7, respectively. The value was found to be specific to the type of callus.

The brightness center, $C_{Y-W}$, was calculated as:

$$C_{Y-W} = \sum Br(Y-W) \times f(Y-W) / \sum f(Y-W)$$

where $f(Y-W)$ shows the frequency of $y-w$ at $Br(Y-W)$.

In order to apply the average brightness center values, $Av(C_{Y-W})$, to callus selection, 50 to 60 images of each of the 8 callus clones were collected in the same fashion. Among them, images of small or irregularly colored calli were removed and 20 images were selected, then the value of $Av(C_{Y-W})$ was calculated. It was found that the callus clone with less than 10 units of $Av(C_{Y-W})$ never regenerated and a proportional relationship between $Av(C_{Y-W})$ and the regeneration frequency of the callus clone, *Fr*, was obtained in the form of the following equation:

$$Fr\ (\%) = 3 \times (Av(C_{Y-W}) - 10).$$

These results suggest that embryogenic calli can be selected from a mixed population of calli since the yellowish compact callus generally shows high embryogenicity. The image analysis using $Br(Y-W)$ proposed here is a useful tool for automatic selection of such calli in a plant factory [74].

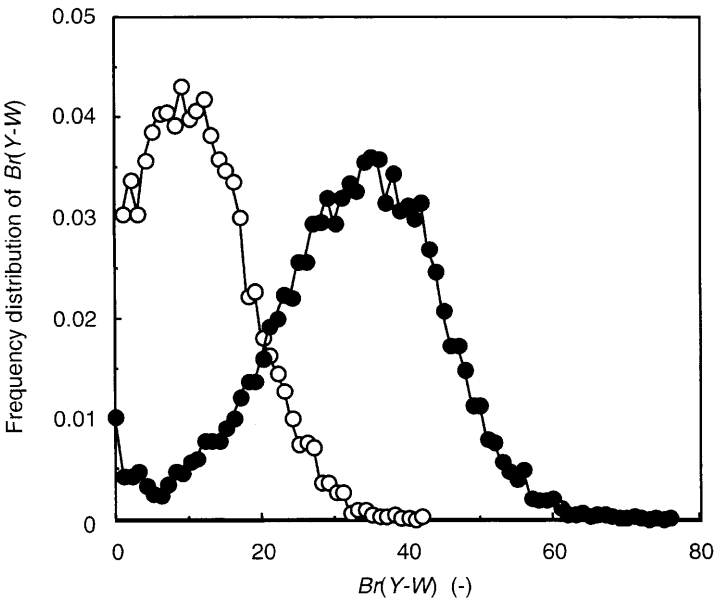


**Fig. 13.** Frequency distribution of $Br(Y-W)$ for compact calli (●) and friable calli (○)

## 4.2 Selection of Embryos

To decide the time to transfer to the next culture stage in plant somatic embryo culture, the classification of embryos and non-embryos using image analysis with artificial neural network (ANN) was developed [76]. In general, the healthy embryos can develop via three morphological types of cells; globular, heart, or torpedo shape. From microscopic observation, human experts can distinguish the embryos from the non-embryos. This is one of the most reliable decision-making modalities. Therefore, if the morphological characteristics of the healthy embryos are correctly extracted from image data and information analysis is also done as well, a computer vision system for screening will be established. ANN is an intelligent technology that can be used for estimation and prediction instead of the human decision; it has an analogy with the human brain. It has been said that ANN could provide solutions by learning complex relationships without requiring the knowledge of the model structure.

Celery embryogenic callus was used. The suspension culture was diluted with water to avoid the overlapping of embryos and poured in a 96-well Petri dish for embryo classification. Digital images from the cell preparations were acquired via an optical microscope equipped with an RGB color CCD video camera. The three channels (red, green, and blue) from the video signal were fed to an image acquisition board installed in an image-processing computer. The acquired image was digitized to a binary image using a binary transformation on the basis of all pixels of brightness level which was the average of the three channel levels. From the vast image analysis database, four parameters (area, ratio of length to width, circularity, and distance dispersion) were selected as morphological characteristics on the basis of the human decision. The structure of an ANN is shown in Fig. 14. Among the four parameters, the use of the first three was satisfactory for the classification between embryos and non-embryos.

After the neural network had been trained with 67 plant cells (23 of embryos and 44 of non-embryos), 33 plant cells (19 of embryos and 14 of non-embryos) were tested to narrow down the parameters extracted from three kinds of embryo or non-embryo for an adequate classification performance by the neural network. The ability of the resultant network with four parameters, area, circularity, ratio of length to width, and distance dispersion was excellent: 89.5% of objects were correctly classified with a corresponding human recognition. Three parameters, area, circularity, and ratio of length to width were necessary for high recognition efficiency, and the distance dispersion could be omitted from parameters without loss of recognition ability. For three kinds of embryo and non-embryo classification, area and circularity provided a relatively large contribution for the correct classification.

Using the ANN model acquired, the total of heart and torpedo embryos in a practical suspension culture was estimated. That is 1140 at the end of the regeneration stage. After the culture was transferred into the maturation stage, it generated 1240 plantlets (Table 3). The ANN results matched highly the ex-

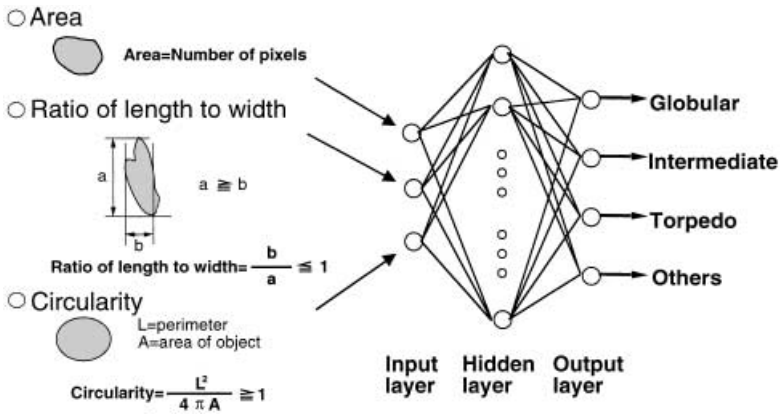


**Fig. 14.** Structure of artificial neural network

**Table 3.** Prediction of plantlet formation by neural network

| Recognized embryos | Globular | Intermediate | Torpedo | Total |
|---|---|---|---|---|
| | 660 | 840 | 300 | 1800 |
| Obtained particles | Abnormal | Plantlet | | Total |
| | 575 | 1240 | | 1816 |

perimental results, thereby indicating a high potential of the image analysis for prediction of plantlets number.

### 4.3 Estimation of Shoot Length

Regenerated callus obtained from bioreactor cultures need to be transferred onto a solid medium and acclimatized under light irradiation so as to obtain healthy plants. In this step, the timing of transfer is different for each plantlet: only plantlets with a long shoot, which confers high photosynthetic ability, should be transferred. Therefore, the development of an automatic selection system using a computer vision system is desired. In the case of the micropropagation of embryogenic rice callus, regenerated rice callus with a shoot of more than 2 cm in length could be transferred from a medium supplemented with sucrose to a medium without sucrose for acclimatization (Fig. 15) [77]. Image analysis was applied for the automatic selection of regenerated rice callus for acclimatization.

Mature rice seeds (*Oryza sativa* L., Sasanishiki) harvested in 1992 were used. After 2- to 3-weeks culture, regenerated callus was transferred onto an acclimatization medium, consisting of MS medium supplemented with 5 mM MES and 4 g/L Gelrite prepared in a plant pot [78]. Incubation was done under the same

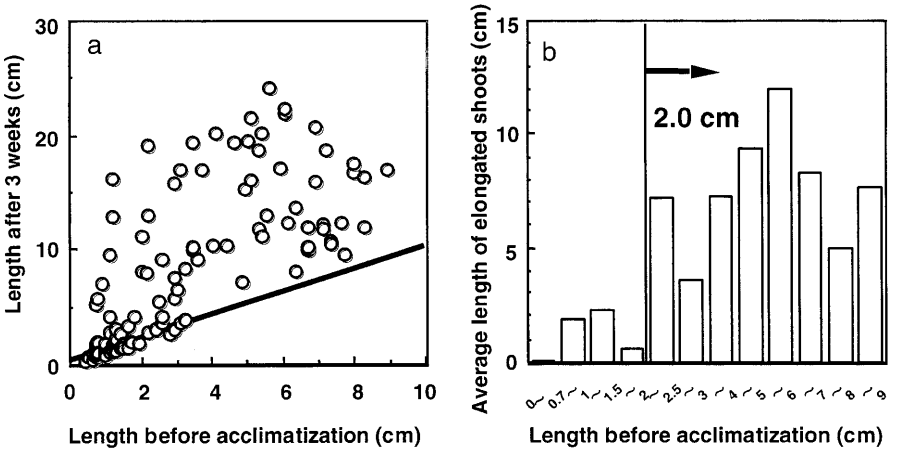


**Fig. 15.** Lengths of elongated shoots after transfer to acclimatization medium. a: For a particular callus; b: for callus in each length range

conditions as those used for regeneration. After 3 weeks, elongated shoots were straightened and the length of elongation was measured. To estimate a shoot length, extraction of the shoot region from the image data is necessary. Therefore, an identification model was constructed in which, for each pixel, one region – shoot, callus, or medium – could be identified. The original image was stored as data sets of R(red), G(green), and B(blue) brightnesses with 256 levels.

For regeneration, one multi-regression analysis (MRA) model and two fuzzy neural network (FNN) models were compared with each other. FNN-B consists of 3 independent models, which are FNNs with 3 input units and 1 output unit. Different grades of fuzzy variables are obtained from the data sets of the i-th pixel in each of the 3 models. On the other hand, FNN-A is comprised of only one model with 3 input and 3 output units. The correctness of the predicted recognition against all data points in the two FNN models was determined. In both FNN models, the recognition correctness of all subjects was remarkably high. The correctness for medium was close to 100 %, and 95 % was obtained for shoot recognition (Table 4).

In order to predict the shoot length, the shoot region was extracted from the ternary image after pretreatment, i. e., such as removal of small, isolated shoot

**Table 4.** Comparison of correctness in FNN-A and FNN-B modeling

| Model | Performance index *J* | Correctness (%) | | | | |
|---|---|---|---|---|---|---|
| | | Shoot | Callus | Medium | All | Average |
| MRA | 0.0657 | – | – | – | – | – |
| FNN-A | 0.0369 | 95.0 | 83.9 | 98.9 | 92.6 | 92.6 |
| FNN-B | 0.0350 | 95.0 | 86.7 | 98.9 | 93.5 | 93.6 |

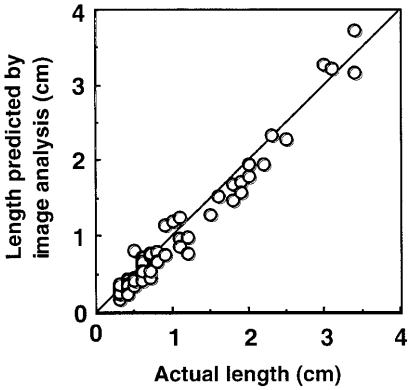


**Fig. 16.** Relationships between actual lengths and lengths obtained by image analysis

regions and filling-up small, isolated non-shoot regions occurring in the shoot region. After thinning and extraction of the longest path, the number of pixels constituting the remaining line was counted.

The actual shoot lengths were measured when the original images were taken. The correlations between the actual lengths and the predicted ones are shown in Fig. 16. Data points plotted on the diagonal line mean that the shoot length was accurately predicted by the image analysis. Almost all data points are seen to be close to the line, demonstrating that the level of accuracy was significantly high. The average error was only 1.3 mm. In Fig. 15, it was shown that a difference of 5 mm should be able to be distinguished. Therefore, it was concluded that the computer-aided image analysis developed here can be useful for the prediction of shoot length and the automatic transfer of regenerated callus to an acclimatization medium.

## 5
## Conclusion and Perspectives

The rearing of embryogenic calli and hairy roots offers a unique opportunity for the large-scale micropropagation of plants. A bioreactor for plant cell, tissue and organ culture, which maintains high levels of mixing and mass transfer but reduces the intensity of shear, is an effective tool in this endeavor. Immobilization has become an important strategy for the removal of shear stress to keep a high regeneration ability of the plant cells in large-scale bioreactor cultivation. In our recent work [79], regeneration frequency of carrot callus was found to be increased by addition of viscous additives in medium. This is a very convenient method for lowering the shear stress. Image analysis with computer devices and robotic systems will become a powerful technology in the optimization of the micropropagation process. An efficient combination of these developing technologies will be a great aid to accelerate the process automation and make it commercially competitive. Lager-scale micropropagation will open up a bright future for the modernization of agriculture.

## References


1. Murashige T (1974) Ann Rev Plant Physiol 25:135
2. Denchev PD, Kuklin AI, Scragg AH (1992) J Biotechnol 26:99
3. Ammirato PV, (1989) Newsl IAAPTC 57:2
4. Redenbaugh K (1990) Hortic Sci 25:251
5. William EG, Maheswaran G (1986) Ann Bot 57:443
6. Chen THH, Thompson BG, Gerson DF (1987) J Ferment Technol 65:353
7. Matsumoto T, Tanaka N (1991) Agric Biol Chem 55:1019
8. Liu CZ, Wang YC, Ouyang F, Ye HC, Li GF (1997) Biotechnol Lett 19:927
9. Flores HE, Hoy MW, Pickard JJ (1987) Trends Biotechnol 5:64
10. Taya M, Yoyama A, Nomura R, Kondo O, Matsui C, Kobayashi T (1989) J Ferment Bioeng 67:31
11. Tepfer D (1984) Cell 37:959
12. Lambert C, Tepfer D (1991) Bio/Technol 9:80
13. Tanaka N, Takao M, Matsumoto T (1994) Plant Tissue Culture Lett 11:191
14. Saitou T, Tachikawa Y, Kamada H, Watanabe M, Harada H (1993) Planta 189:590
15. Hamill JD, Prescott A, Martin C (1987) Plant Mol Biol 9:573
16. Hooker BS, Lee JM, An G (1989) Enzyme Microb Technol 11:484
17. Lindsey K, Yeoman MM, Black GM, Mavituna F (1983) FEBS Lett 155:143
18. Nakajima HK, Sonomoto H, Morikawa H, Sato F, Ichimura K, Yamada Y, Tanaka A (1986) Appl Microbiol Biotechnol 24:266
19. Facchini PJ, DiCosmo F (1990) Appl Microbiol Biotechnol 33:36
20. Hulst AC, Meyer MMT, Breteler H, Tramper J (1989) Appl Microbiol Biotechnol 30:18
21. Suehara K, Kohketsu K, Uozumi N, Kobauashi T (1995) J Ferment Bioeng 79:585
22. Nagamori E, Honda H, Kobayashi T (1999) J Biosic Bioeng 88:226
23. Chi CM, Vits H, Staba EJ, Cooke TJ, Hu WS (1994) Biotechnol Bioeng 44:368
24. Prenosil JE, Pesersen H (1983) Enzyme Microbiol Technol 5:323
25. Scagg AH, Fowler WM (1985) In: Vasil I (ed) Cell culture and somatic cell genesis of plants. Academic Press, London, p 103
26. Panda AK, Saroj M, Bisaria VS, Bhojwani SS (1989) Enzyme Microb Technol 11:386
27. Doran PM (1993) Adv Biochemical Eng Biotechnol 48:117
28. Payne Ak, Shuler ML, Brodelius P (1987) In: Lydersen BK (ed) Large scale cell culture technology. Hansen Publishers, New York. p 193
29. Hooker BS, Lee JM, An G (1990) Biotechnol Bioeng 35:296
30. Treat WJ, Engler CR, Soltes EJ (1989) Biotechnol Bioeng 34:1191
31. Zhong JJ, Yu JT, Yoshida T (1995) World J Microbiol Biotechnol 11:461
32. Chisti Y, Moo-Young M (1993) J Chem Technol Biotechnol 58:331
33. Jolicoeur M, Chavarie C, Carreau PJ, Archambault J (1992) Biotechnol Bioeng 39:511
34. Archambault J, Williams RD, Lavoie L, Pepin MF, Chavarie C (1994) Biotechnol Bioeng 44:930
35. Wang SJ, Zhong JJ (1996) Biotechnol Bioeng 51:511
36. Wang SJ, Zhong JJ (1996) Biotechnol Bioeng 51:520
37. Styer DJ (1985) In: Henke RR, Hughes KW (eds) Tissue culture in forestry and agriculture. Plenum Publishing Corp, New York, p 117
38. Ammirato PV, Styer DJ (1985) In : Zaitlin M, Day P, Hollaender A (eds) Biotechnology in plant Science: relevance to agriculture in eighties. Academic Press, New York, p 117
39. Rodriguez-Mendiola MA, Stafford A, Cresswell R, Arias-Castto C (1991) Enzyme Microbiol Technol 13:697
40. Liu CZ, Wang YC, Zhao B, Guo C, Ouyang F, Ye HC, Li GF (1999) In vitro Cell Dev Biol Plant 35:271
41. Ramakrishnan D, Curtis WR (1994) In: Furusaki S, Ryu D (eds) Advances in plant biotechnology: production of secondary metabolites. Elsevier, Amsterdam, p 281
42. Whitney PJ (1992) Enzyme Microb Technol 14:13
43. Yu SX, Doran PM (1994) Biotechnol Bioeng 44:880

44. Taya M, Yoyama A, Kondo O, Kobayashi T (1989) J Chem Eng Jpn 22:84
45. Nagai N, Kurosawa I, Kanda T, Okazaki M (1994) J Ferment Bioeng 77:212–214
46. Kondo O, Honda H, Taya M, Kobayashi T (1990) Appl Microbiol Biotechnol 33:291
47. Uozumi N, Kohketsu K, Kobayashi T (1993) J Chem Tech Biotechnol 57:155
48. Murashige T (1978) In: Thorpe T (ed) Frontiers of plant tissue culture. International Association of Plant Tissue Culture, Calgary, Canada, p 15
49. Kitto SL, Janick J (1985) J Amer Soc Hort Sci 110:277
50. Redenbaugh K, Paasch BD, Nichol JW, Kossler ME, Viss PR, Walker KA (1986) Bio/technol 4:797
51. Redenbaugh K, Slade D, Viss P, Fujii J (1987) HortSci 22:803
52. Uozumi N, Kobayashi T (1995) Biotechnol Agricult Forestry 30:170
53. Uozumi N, Nakashimada Y, Kato Y, Kobayashi T (1992) J Ferment Bioeng 74:21
54. Nakashimada Y, Uozumi N, Kobayashi T (1994) J Ferment Bioeng 77:178
55. Uozumi N, Asano Y, Kobayashi T (1994) Plant Cell Tissue Organ Cult 36:183
56. Nakashimada Y, Uozumi N, Kobayashi T (1995) J Ferment Bioeng 79:458
57. Nakashimada Y, Uozumi N, Kobayashi T (1996) J Ferment Bioeng 81:87
58. Uozumi N, Ohtake Y, Nakashimada Y, Morikawa Y, Tanaka N, Kobayashi T (1996) J Ferment Bioeng 81:374
59. Honda H, Hattori T, Uozumi N, Kobayashi T, Kato Y, Hiraoka S (1997) J Chem Eng Jpn 30:179
60. Brodelius P, Deus B, Mosbach K, Zenk MH (1979) FEBS 103:93
61. Majerus F, Pareilleux A (1986) Plant Cell Rep 5:302
62. Rhodes MJC (1985) Top Enzyme Ferment Bioeng 10:51–87
63. Seuhara K, Nagamori E, Honda H, Uozumi N, Kobayashi T (1998) J Chem Eng Jpn 31:613
64. Nakashima T, Kyotani S, Izumoto E, Fukuda, H (1990) J Ferment Bioeng 70:85
65. Wakisaka Y, Segawa T, Imamura K, Sakiyama T, Nakanishi K (1998) J Ferment Bioeng 85:488
66. Rhodes MJC, Smith JI, Robins RJ (1987) Appl Microbiol Biotechnol 26:28
67. Furuya T, Orihara Y, Koge K, Tsuda Y (1990) Plant Cell Rep 9:125
68. Corchete P, Yeoman MM (1989) Plant Cell Rep 8:128
69. Chu CC, Wang CC, Sun CS, Hsu C, Chu KC, Bi FY (1975) Sci Sin 18:659
70. Moon KH, Honda H, Kobayashi T (1999) J Biosci Bioeng 87:661
71. Liu CZ, Moon KH, Honda H, Kobayashi T (2000) Biochem Eng J 4:169
72. Kobayashi H, Okii M, Hirosawa T (1992) Jpn J Breed 42:583
73. Liu CZ, Moon KH, Honda H, Kobayashi T (2001) J Biosci Bioeng 91:76
74. Raghava-Ram NV, Nabors MW (1985) Plant Cell Tissue Organ Cult 4:241
75. Honda H, Ito T, Yamada J, Hanai T, Matsuoka M, Kobayashi T (1999) J Biosci Bioeng 87:700
76. Uozumi N, Yoshino T, Shiotani S, Seuhara K, Arai F, Fukuda T, Kobayashi T (1993) J Ferment Bioeng 76:505
77. Honda H, Takikawa N, Noguchi H, Hanai T, Kobayashi T (1997) J Ferment Bioeng 84:342
78. Nursery Technology Co. (1993) Kenkyusho houkoku, Nursery Technology Co., Tokyo
79. Nagamori E, Omote M, Honda H, Kobayashi T (2001) J Biosci Bioeng 91:283

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# Characterization and Application of Plant Hairy Roots Endowed with Photosynthetic Functions

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The scope of this review includes the physiological properties of the hairy roots induced by light irradiation and the kinetic analysis considering the effects of light intensity on hairy root cultures. The cell lines of photomixotrophic and photoautotrophic hairy roots of pak-bung are established from heterotrophic ones by improving the photosynthetic ability of hairy roots through acclimation cultures under light irradiation. Comparisons of physiological properties of derived photoautotrophic cell line with photomixotrophic and heterotrophic ones are also made through histological examination. Moreover, the effect of photosynthesis inhibitor on the photoautotrophic growth of the hairy roots is described. By elucidating the influences of light intensity on growth and chlorophyll formation of photoautotrophic and photomixotrophic hairy roots, a kinetic model was applied to describe the hairy root growth and chlorophyll formation of these cell lines of hairy roots.

**Keywords.** Plant cell culture, Pak-bung hairy roots, Photoautotrophic culture, Light irradiation



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## List of Abbreviations and Symbols

| | |
|---|---|
| 0 (subscript) | Value at initial stage of culture |
| $A_A$ | Average APx activity in plant materials |
| $A_{Chl}$ | Amount of Chl on culture volume basis |
| $A_G$ | Average GPx activity in plant materials |
| $A_{POD}$ | Average POD activity in plant materials |
| APx | Ascorbate peroxidase |
| $A_R$ | Average RubisCO activity in plant materials |
| $A_{SOD}$ | Average SOD activity in plant materials |
| $C_{Chl}$ | Average Chl content in plant materials |
| $\hat{C}_{Chl}$ | Chl content in hairy roots at given distance from root tip of hairy roots |
| $\hat{C}_{Chl,S}$ | Saturated Chl content in hairy roots at given distance from root tip of hairy roots |
| $\overline{C}_{Chl}$ | Chl content in segments at given distance from root tip of hairy roots |
| $C_F$ | Fructose concentration in medium |
| $C_S$ | Sucrose concentration in medium |
| $\hat{C}^i$ | Chl concentration at inside of chloroplast in cells existing at given distance from root tip of hairy roots |
| $C_{min}$ | Critical concentration of $O_2$ |
| $\hat{C}^0$ | Chl concentration at outside of chloroplast in cells existing at given distance from root tip of hairy roots |
| Chl | Chlorophyll |
| $D$ | Diameter of hairy roots |
| DCMU | 3-(3,4-dichlorophenyl)-1,1-dimethyl urea |
| DW | Dry cell weight |
| F (subscript) | Final value |
| FW | Fresh cell weight |
| GP | Growing point |
| GPx | Guaiacoal peroxidase |

| | |
|---|---|
| $i$ | Number of decayed GPs in Eq. (7) |
| $I$ | Incident light intensity |
| I (subscript) | Initial value |
| IAA | Indole acetic acid |
| $k_C$ | Apparent permeation rate constant through envelope of chloroplast in Eq. (13) |
| $k_D$ | Decay rate constant in Eq. (8) |
| $K_F$ | Saturation constant in Eq. (18) |
| $K_I$ | Saturation constant in Eq. (19) |
| $l$ | Distance from tip of hairy roots |
| $L$ | Length of hairy roots |
| $L_B$ | Interval between neighboring branches |
| $L_{BD}$ | Interval between neighboring branches cultivated in the dark |
| $L_G$ | Length of growing point |
| $L_{I,n_b}$ | Length of hairy roots at initial stage of $n_b$-th branching |
| $m$ | Number of decayed GPs between $n_b$-th and $(n_b+1)$-th branching in Eq. (7) |
| $m_R$ | Maintenance energy based on $O_2$ uptake rate |
| $m_S$ | Maintenance energy based on sugar consumption rate |
| $M$ | Overall amount of chlorophyll in hairy roots |
| MS | Murashige-Skoog |
| $n$ | Number of lateral roots per unit length of main roots |
| $n_b$ | Number of branching |
| $n_b$ (subscript) | Value at $n_b$-th branching |
| $N$ | Number of GPs |
| $N_b$ | Number of budding roots per unit length of the main roots |
| $N_{Chl}$ | Density of chloroplasts in hairy roots at given distance from root tip of hairy roots |
| $N_{F,n_b}$ | Number of GPs at final stage of $n_b$-th branching |
| $N_{I,n_b}$ | Number of GPs at initial stage of $n_b$-th branching |
| POD | Peroxidase |
| $R_G$ | Elongation rate of growing points |
| $R_{GI}$ | Initial elongation rate of growing points |
| S (subscript) | Saturated value |
| SOD | Superoxide dismutase |
| $t$ | Culture time |
| $t_{I,n_b}$ | Culture time at initial stage of $n_b$-th branching |
| $V$ | Volume of medium |
| $W_C$ | Water content of hairy roots |
| $X$ | Concentration of root mass |
| $Y^*$ | True cell yield |
| $\Theta$ | Constant in Eq. (27) |
| $\theta$ | Cellular age |
| $\alpha$ | Viability |
| $\beta$ | Volume of chloroplast per unit volume of single cell |
| $\Delta t$ | Period until next branching of GP in Eq. (4) |
| $\chi$ | Constant in Eq. (20) |

| | |
|---|---|
| $\sigma$ | Constant in Eq. (20) |
| $\varepsilon$ | Constant in Eq. (22) |
| $\phi$ | Constant in Eq. (22) |
| $\gamma$ | Constant in Eq. (23) |
| $\eta$ | Constant in Eq. (24) |
| $\iota$ | Constant in Eq. (26) |
| $v_{CF}$ | Carbon fixation rate per dry cell weight based on carbon dioxide |
| $v_{SF}$ | Carbon fixation rate per dry cell weight based on sucrose |
| $\mu$ | Specific elongation rate |
| $\mu_{Fmax}$ | Maximum specific elongation rate of photomixotrophic hairy roots |
| $\mu_{Imax}$ | Maximum specific elongation rate of photoautotrophic hairy roots |
| $\varrho$ | Density of hairy roots |
| $\omega_i$ | Time at *i*-th GP decay |

## 1 Introduction

Since the 1930s when the first in vitro cultures were established, a great deal of advances have been achieved with respect to plant biotechnology including application of plant cell culture and molecular genetics to crop improvement, preservation of genetic diversity, propagation of elite plants, and metabolite production [1, 2]. Although plant cell culture appears to be very advantageous technique, some problems remain to be solved for practical uses. To overcome these problems, a lot of studies have been conducted concerning cell line improvement, medium optimization, genetic engineering, culture operation system, cell immobilization, and so on [3, 4].

Transformed plant roots, also called hairy roots, are caused by infection with the soil bacterium *Agrobacterium rhizogenes*, and are associated with the integration of transfer-DNA region on a bacterial root-inducing plasmid into chromosomal DNA in plant cells [5]. Hairy roots exhibit fast growth in the absence of exogenous plant growth regulators (phytohormones) without losing their differentiated phenotype. They also have high biosynthetic capacity, in particular root-inherent metabolites as found in original roots in vivo. In contrast with the instability and variability of unorganized plant cell suspensions, hairy roots are genetically stable. The transformation system seems to facilitate further genetic manipulation and can contribute to the development of cultures with outstanding characteristics. Therefore, for the last decade, with a significant advancement in the techniques of plant tissue cultures, hairy roots have been regarded as promising materials in plant cell cultures. Many researchers have made assiduous attempts to produce desired metabolites derived from the hairy roots [6].

In recent years, it has been shown that hairy roots are responsive to physical stimuli such as exposure to light. Flores and Curtis [7] and Sauerwein et al. [8]

have reported that some hairy roots, including *Acmella oppositifolia*, *Datura stramonium*, and *Lippia dulcis*, turned green when exposed to light, while maintaining their morphologies in branched fibrous roots. They also demonstrated that the green hairy roots had metabolite productivities which were distinct from their respective original ones: e.g., alkaloid production by *D. stramonium* hairy roots [7] and terpene production by *L. dulcis* hairy roots [8]. These findings indicate that the exposure of hairy roots to light leads to alternations in the biosynthetic potentials of hairy roots. As in the case of cell suspension cultures, the temperature profoundly affected on metabolite production and growth of hairy root cultures [9–11]. Most studies, which have been reported hitherto, intended to achieve the optimal growth and metabolite productivity. However, physiological properties of hairy roots caused by environmental conditions have been hardly investigated in engineering aspects.

We reported the induction of heterotrophic hairy roots from the pak-bung (*Ipomoea aquatica*) plant and the production of superoxide dismutase (EC 1.15.1.1, SOD) and peroxidase (EC 1.11.1.7, POD) by culture of hairy roots [12, 13]. In the present review, the photomixotrophic green hairy roots of pak-bung were developed via subcultures of the original white hairy roots under continuous light conditions as shown in Fig. 1 [14]. And the investigation of the growth properties and enzyme (SOD and POD) activities of pak-bung green hairy roots was conducted. In addition, from the viewpoint of extending availability of hairy roots, the establishment of a photoautotrophic cell line of pak-bung hairy roots was attempted [15], and the characterization of the photoautotrophic hairy roots was discussed in comparison with photomixotrophic and heterotrophic ones. To clarify the properties of growth and chlorophyll, Chl, formation of these cells, kinetic analyses were examined in terms of light intensity. Moreover, photoautotrophic cultures of hairy roots were carried out under conditions where captured energy by the cells would be a rate-limiting factor for root elongation. Relationship between growth potential of photoautotrophic hairy roots and energy acquired by photosynthesis in the cells was discussed in terms of maintenance energy.

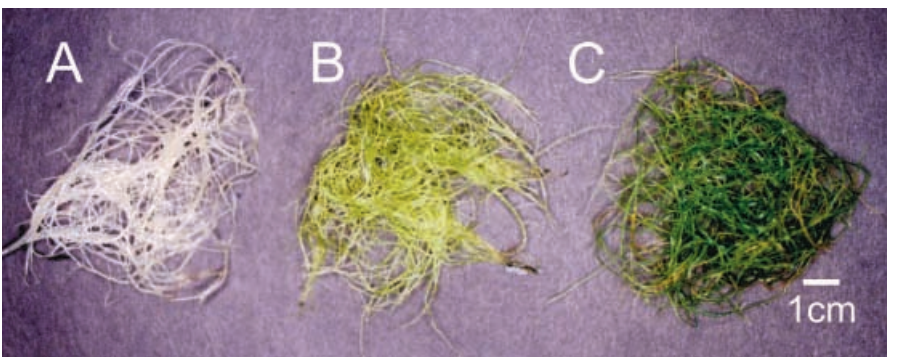


**Fig. 1 A–C.** Photograph of pak-bung hairy roots: **A** heterotrophic hairy roots; **B** photomixotrophic hairy roots; **C** photoautotrophic hairy roots

## 2
## Experimental Procedures

### 2.1
### Plant Materials

Transformed roots of pak-bung induced by means of *A. rhizogenes* A4 infection [12] were used as the original hairy roots (white). Subcultures of the heterotrophic hairy roots were conducted every 14 days in 200-$cm^3$ Erlenmeyer flasks containing 100 $cm^3$ Murashige-Skoog, MS, liquid medium [16] with 20 kg $m^{-3}$ sucrose. The flasks were incubated at 25 °C in darkness on a gyratory shaker with 100 rpm shaking.

For the establishment of photomixotrophic hairy roots, the original heterotrophic hairy roots were subcultured every two weeks at 25 °C in MS liquid medium containing 30 kg $m^{-3}$ sucrose and no phytohormone under continuous light using a set of four white fluorescent lamps. After a series of subcultures for about six months, the acclimated hairy roots (green) were subjected to experimentation. The photomixotrophic hairy roots of pak-bung, which have been established as described above were maintained by subculturing every 14 days at 25 °C in MS liquid medium containing 30 kg $m^{-3}$ sucrose under continuous light irradiation.

To derive a photoautotrophic cell line, the photomixotrophic hairy roots were used as an origin, and the procedure of the establishment is described in Sect. 3.2.

The photoautotrophic hairy roots were maintained through subcultures using conical glass flasks containing sugar-free MS liquid media at 28 days' interval under the conditions of 5.0 % $CO_2$ and continuous light irradiation.

Specimens of parent plants of pak-bung were obtained from whole plants grown outdoors for about 1–2 months.

### 2.2
### Culture Methods and Conditions

Through the experiments for characterization of photomixotrophic hairy roots, cultures were carried out at 25 °C using 125-$cm^3$ conical glass flasks containing 50 $cm^3$ MS liquid medium (pH 5.7) with 20 kg $m^{-3}$ fructose and no phytohormone. The hairy roots were grown with an inoculum of about 0.2 g fresh weight, FW, in flasks shaken at 100 rpm on a rotary shaker located beneath the lamps.

The inocula for these experiments were prepared by preculturing green hairy roots in MS liquid medium for 14 days at incident light intensity, $I$, of 11.1 W $m^{-2}$ under the same conditions as mentioned above.

Unless otherwise noted, the flasks were continuously illuminated at a light intensity of $I = 11.1$ W $m^{-2}$ using a set of four lamps. When necessary, the light intensity was varied by changing the distance between the flasks and lamps and by partially shading the lamps with black adhesive tape.

The photoautotrophic cultures were carried out at 25 °C using 250-$cm^3$ flat, oblong glass flasks containing 50 $cm^3$ of MS liquid medium. The hairy roots

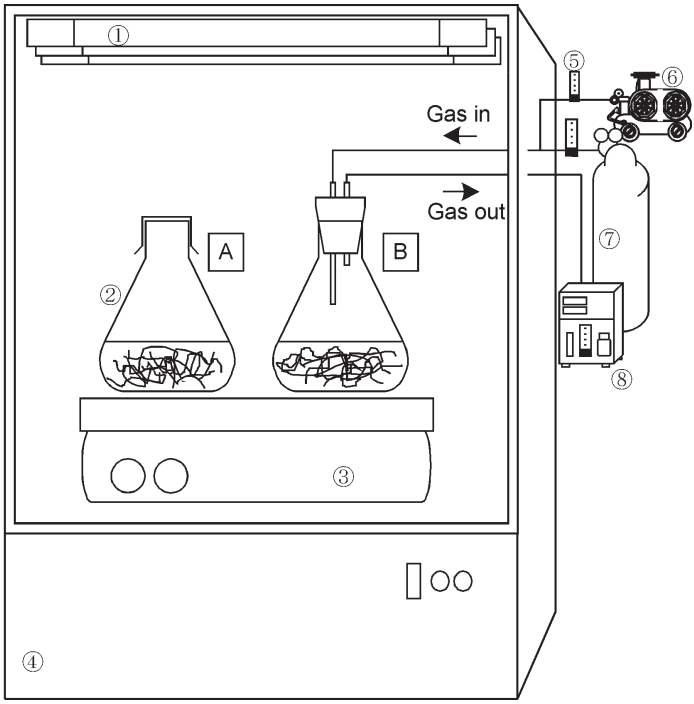


**Fig. 2.** Schematic drawing of photo-incubator. A: Plain air conditions, B: $CO_2$-enriched air conditions. ① White fluorescent lamp, ② Conical flask, ③ Rotary shaker, ④ Incubator, ⑤ Gas flow meter, ⑥ Air compressor, ⑦ $CO_2$ gas cylinder, ⑧ Gas analyzer

were grown with an inoculum of 0.5 g-FW in the flasks shaken on a rotary shaker and illuminated at various incident light intensities with 20 W white fluorescent as shown in Fig. 2. For photoautotrophic cultures, fructose was excluded from the medium and $CO_2$-enriched air (5.0 % $CO_2$) was made to flow into the culture flasks at a flow rate of 0.6 $dm^3\ h^{-1}$. The light intensity was adjusted by changing the distance between the lamps and flasks.

In experiments to evaluate the elongation rate of growing points at root tip meristems, the hairy roots were cultivated under the same conditions with Petri dishes (9 cm in diameter) containing solidified MS medium.

The elongation rates of growing points, $R_G$, at root tip meristems were evaluated by measuring the change in the positions of root tips on the MS solid medium every day during culture time of 7 – 14 days. The data were average values obtained from the measurements with respect to about 15 root tips.

To evaluate the viability of growing points, the hairy roots were cultivated on sugar-free MS plates at various incident light intensities and the root tips elongating more than 1 mm per day were regarded as the viable growing points.

To determine the resumption potential of the hairy roots, three pieces of the main roots, which were randomly picked up from the liquid cultures at prescribed time, were transferred on the MS solid medium with 20 kg $m^{-3}$ sucrose

and kept at 25 °C in the dark for 20 days. Lateral roots budding on the respective main roots were numerated as a criterion of the resumption potential of the examined roots. The data were expressed as an average value of the number of budding roots per unit length of the main root, $N_b$.

The hairy roots, which had various lengths from root tips of $L = 5.0 \times 10^{-3}$ m to $9.0 \times 10^{-2}$ m, were prepared and placed on MS solid medium after their lateral roots were cut off. The dishes were incubated in a chamber at 5.0% $CO_2$ concentration and incident light intensities were kept at 11 W $m^{-2}$ and 20 W $m^{-2}$ on the level of the medium surface.

### 2.3 Analytical Methods

The hairy roots were harvested by paper filtration and rinsed with a large amount of water for root mass measurement. The dry weight, DW, of the hairy roots was gravimetrically measured after drying the harvested roots at 80 °C for 24 h.

The Chl in the hairy roots and parent plant organs was extracted with a mixture of 80% acetone and 20% phosphate buffer (2.5 mol $m^{-3}$, pH 7.8), and analyzed according to the method described by Porra et al. [17]. The activities of SOD and POD in the hairy roots and parent plant organs were spectrophotometrically determined using ferricytochrome C [18] and *o*-aminophenol [19], respectively. Fructose in the medium was analyzed by the Somogyi-Nelson method [20] or was determined using an HPLC with a refractive index detector. Light intensity was measured with a thermopile on the outer walls of flasks at the level of the medium surface or at the top of the Petri dish, and expressed as the mean of values determined at several positions.

To investigate the influence of a photosynthesis inhibitor on the growth of the hairy roots, 0–10 mmol $m^{-3}$ of 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea, DCMU, was supplemented in the medium according to the procedure described by Horn et al. [21]. The dishes were incubated in a chamber under the varied $CO_2$ concentrations of 0.03–8.5%.

The activity of ribulose-1, 5-bisphosphate carboxylase/oxygenase, RubisCO, (EC 4.1.1.39) in the cells was assayed according to the procedures of Racker [22]. The activities of ascorbate peroxidase, APx, (EC 1.11.1.11) and guaiacoal peroxidase, GPx, (EC 1.11.1.7) were determined using ascorbate and pyrogallol as substrates, respectively [23, 24]. The apparent $CO_2$ fixation rate of the hairy roots was evaluated as follows using the apparatus shown in Fig. 2. Air with 5.0% $CO_2$ was introduced into 250-$cm^3$ flat, oblong glass flasks containing the hairy roots at a flow rate of 150 $cm^3$ $h^{-1}$ and $CO_2$ concentration in the outlet gas was measured at 25 °C with a $CO_2$ gas analyzer. The apparent $CO_2$ fixation rate was calculated from the differences between $CO_2$ concentrations in the inlet and outlet gas phases determined in the tests in the dark and under light irradiation of $I = 11$ W $m^{-2}$.

The content of indole-3-acetic acid, IAA, in cells was determined according to the fluorometric analysis described by Kamisaka and Larsen [25].

### 2.4 Microscopic Observations

For optical microscopy, hairy root specimens were fixed with 3% formaldehyde in 50 mol $m^{-3}$ phosphate buffer (pH 6.8) for 1 h at ambient temperature. Cross-sections of the specimen were prepared using a sliding microtome equipped with a specimen-freezing stage. The sections were examined with microscopes under conventional or fluorescent light (excitation wavelengths of 520 – 550 nm and emission wavelength of > 580 nm).

For electron microscopy, hairy root specimens were cut into small segments (ca. 5 mm in length). The segments were fixed with 4% glutaraldehyde in 50 mol $m^{-3}$ phosphate buffer (pH 6.8) for 2 h at ambient temperature, followed by post-fixation with 2% osmium tetroxide in the same buffer for 1 h at 4 °C, and otherwise fixed with 1% glutaraldehyde in 50 mol $m^{-3}$ piperazene-*N,N′*-bis(2-ethanesulfonic acid), PIPES, buffer (pH 7.6) for 2 h at ambient temperature, followed by postfixation with 4% osmium tetroxide in the PIPES buffer for 2 h at 4 °C. After dehydration in a graded ethanol series, the segments were grounded in Spurr's resin [26]. Thin sections were prepared with an ultramicrotome, stained with uranyl acetate and lead citrate in turn, and then examined with a JEM 100 S transmission electron microscope.

## 3 Photosynthetic Ability of Pak-Bung Hairy Roots Induced by Light Irradiation

### 3.1 Chlorophyll Content and Enzyme Activities in Photomixotrophic Hairy Roots

The chlorophyll content, $C_{Chl}$, and enzyme activities of superoxide dismutase, and peroxidase, $A_{SOD}$, and $A_{POD}$, in the photomixotrophic green hairy roots of pak-bung grown in light ($I = 11.1$ W $m^{-2}$) were compared with those in heterotrophic white hairy roots (original roots) grown in the dark and parent plants grown outdoors. As shown in Table 1, a significant amount of Chl was found in the green hairy roots, whereas Chl was not detected in the original hairy roots or parent plant roots. The $C_{Chl}$ value in the green hairy roots cultivated for 21 days was 2.3 g $(kg\text{-}DW)^{-1}$, which was a similar order of magnitude to that found in parent plant stems although lower than that in parent plant leaves. Thus, exposure of pak-bung hairy roots to light irradiation resulted in the greening of the roots and the formation of Chl.

With respect to the production of SOD and POD, green hairy roots showed sufficiently high activities, compared with those in the original hairy roots and parent plant leaves with the highest activities among the organs tested. The $A_{SOD}$ and $A_{POD}$ values in the green hairy roots were enhanced about 3.1-fold and 1.7-fold over those in the original hairy roots, respectively, and about 7.3-fold and 4.3-fold over those in parent plant leaves, respectively.

Moreover, these enzyme activities declined along with the decreased Chl content when the green hairy roots were grown in the dark (data not shown). It

**Table 1.** Chl content and enzyme (SOD and POD) activities in hairy roots and in parent plant organs of pak-bung

| Plant materials | $C_{Chl}$ [g (kg-DW)$^{-1}$] | $A_{SOD}$ [$10^7$ U (kg-DW)$^{-1}$] | $A_{POD}$ [$10^6$ U (kg-DW)$^{-1}$] |
|---|---|---|---|
| Green hairy roots[a] | 2.3 | 2.2 | 4.3 |
| White hairy roots[b] | nil | 0.7 | 2.5 |
| Parent plant | | | |
| Leaves | 19.2 | 0.3 | 1.0 |
| Stems | 3.6 | 0.1 | 0.6 |
| Roots | nil | 0.1 | 0.3 |

[a] Green (photomixotrophic) hairy roots were grown in MS liquid medium for 21 days in light at $I = 11.1$ W m$^{-2}$.
[b] White hairy roots (original roots) were grown in MS liquid medium for 21 days in the dark.

is well known that SOD and POD have important roles in the scavenging of the toxic oxidant species superoxide and peroxide in cells by catalyzing the following reactions [27].

For SOD:

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

and for POD:

$$H_2O_2 + AH_2 \rightarrow 2H_2O + A \text{ (A: electron donor)} \quad (2)$$

According to Asada [28], the production of these enzymes is associated with photochemical reactions in chloroplasts because chloroplasts are involved in the formation of toxic oxidants such as $O_2^{\bullet-}$ and $H_2O_2$. It has been reported that a photosynthetic organism, *Euglena gracilis*, expressed higher SOD activity in a light-irradiated culture than in a dark culture [29]. Thus the enhanced activities of SOD and POD in pak-bung green hairy roots under light exposure may be closely linked to the formation of Chl and the development of a thylakoid membrane system in the cells as described in the later session.

In general, hairy roots are characterized as an underground plant organ (i.e., roots) and thus they exclusively produce secondary metabolites found in the roots of field-grown plants. It was recently reported that some hairy roots acquire certain functions of above ground plant organs (i.e., stems and leaves) in response to light illumination associated with greening in color. Table 2 summarizes the Chl contents of green hairy roots of various plants exposed to light. Chl content values are available for *A. oppositifolia* (85.7–178.6 µg-Chl (kg-FW)$^{-1}$) and *Hyoscyamus albus* (3.3 mg-Chl (kg-FW)$^{-1}$) hairy roots. In the present study, pak-bung green hairy roots were found to possess a Chl content of 1.1–2.3 g (kg-DW)$^{-1}$, corresponding to about 120–260 mg-Chl (kg-FW)$^{-1}$. The Chl content of pak-bung green hairy roots was relatively high compared with that in the above two green hairy roots.

**Table 2.** Chl contents of green hairy roots derived from various plants

| Hairy roots | Medium and light conditions | Chl content | Product tested | Ref. |
|---|---|---|---|---|
| *Acmella oppositifolia* | B5 + 0 – 3% sucrose, continuous light at 70 E $(m^2 \cdot s)^{-1}$ | 85.7–178.6 μg $(g\text{-}FW)^{-1}$ | Polyacetylene | 7, 34 |
| *Bidens sulphureus* | MS + 0.5 – 2% sucrose | – | Polyacetylene | 30, 31 |
| *Datura stramonium* | B5 + 0 – 3% sucrose, continuous light at 70 E $(m^2 \cdot s)^{-1}$ | – | Alkaloids | 7, 34 |
| *Digitalis lanata* | 1/2MS, 16-h light a day at 5, 000 lx | – | Cardenolides | 32 |
| *Hyoscyamus albus* | 1/2MS + 3% sucrose, 16-h light a day | 3.3 μg $(g\text{-}FW)^{-1}$ | Alkaloids | 33 |
| *Lippia dulsis* | MS + 2% sucrose, 16-h light a day at 70 E $(m^2 \cdot s)^{-1}$ | | Terpenes | 8 |
| *Ipomoea aquatica* | MS + 2% fructose, continuous light at 11.1 W $m^{-2}$ | 1.1–2.3 mg $(g\text{-}DW)^{-1}$ | SOD, POD | 14 |
| (pak-bung) | | (120–260 μg $(g\text{-}FW)^{-1}$) | | |

B5: Gamborg's B5 medium, MS: Murashige-Skoog medium, 1/2MS: MS medium with inorganic elements at half strength. The following relationship describes the conversion to light intensity units: 1000 lx = 13 E $(m^2\ s)^{-1}$ = 3.4 W $m^{-2}$.

### 3.2
### Development of Photoautotrophic Hairy Roots

To derive a cell line of photoautotrophic hairy roots, successive cultures of the photomixotrophic hairy roots were performed under the conditions of plain air (0.03% $CO_2$) and $CO_2$-enriched air (3.0% $CO_2$), and the initial concentration of sucrose, $C_{SI}$, as a carbon source was lowered in a stepwise manner, that is, $C_{SI} = 10$ (1st stage for 0–14 days), 5.0 (2nd stage for 14–28 days), 2.5 (3rd stage for 28–42 days), and 0 kg $m^{-3}$ (4th stage for 42–56 days).

In the case of culture under plain air, as shown in Fig. 3A, both the Chl content, $C_{Chl}$, and RubisCO activity, $A_R$, were kept at appreciable levels as long as sucrose was included in the medium (1st to 3rd stages). While being kept in sucrose-free medium (4th stage), however, the hairy roots turned brown and the values of $C_{Chl}$ and $A_R$ were ultimately extinguished.

On the other hand, in the cultures under 3.0% $CO_2$ atmosphere, the values of $C_{Chl}$ and $A_R$ in the hairy roots increased at every culture stage accompanying the decrease in the initial sucrose concentration in the medium as shown in Fig. 3B. At the end of the 2nd and 3rd stages, the sugars (sucrose, glucose, and fructose) were completely consumed in the medium (data not shown). It was thus considered that the hairy roots were acclimating to the photoautotrophic condition

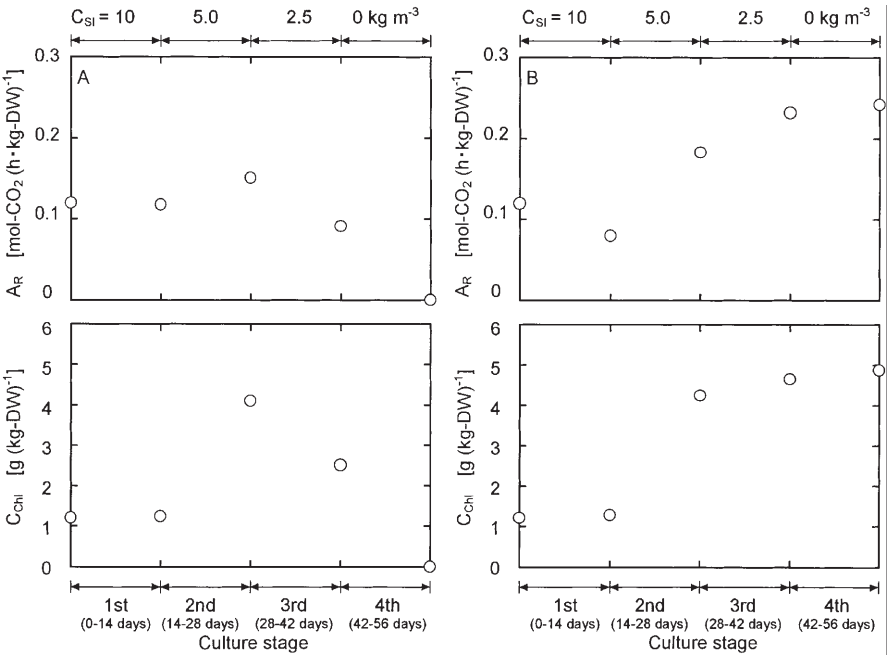


**Fig. 3A, B.** Changes in RubisCO activity and Chl content during acclimation cultures of photomixotrophic pak-bung hairy roots to photoautotrophy. Each culture was carried out using the conical flasks illuminated at $I = 11$ W $m^{-2}$ and shaken at 100 rpm with: **A** plain air (0.03% $CO_2$) supply; **B** 3.0% $CO_2$-enriched air supply

with enhancement of Chl formation and RubisCO induction during the 2nd and 3rd stages. At the end of the 4th stage during which hairy roots were cultivated in the sucrose-free medium, the Chl content and RubisCO activity reached $C_{Chl} = 4.9$ g (kg-DW)$^{-1}$ and $A_R = 0.24$ mol-$CO_2$ (h · kg-DW)$^{-1}$, respectively, which were 4.1- and 2.0-fold higher than the original photomixotrophic hairy roots.

Flores et al. [34] reported that RubisCO activity of *A. oppositifolia* hairy roots increased with decreasing sucrose concentration during acclimating cultures for photoautotrophy, and RubisCO activity of the hairy roots cultivated with sugar-free medium was 1.7 times that cultivated with medium containing 30 kg m$^{-3}$ sucrose.

Photoautotrophic cell lines of plants, that are able to grow with $CO_2$ fixation through photosynthetic reactions in the absence of any organic carbon sources, have been established accompanying the reinforcement of their photosynthetic potentials by means of modifying culture conditions. The photosynthetic potentials of in vitro cultured plant cells are known to be affected by environment factors such as sugar concentration in a medium, $CO_2$ concentration in gas phase and light quantity furnished to the cells. Groß et al. [35] indicated that the activity of ribulose-1, 5-bisphosphate carboxylase/oxygenase, a key enzyme in $CO_2$ fixation in photosynthesis, reduced with the administration of sugar to a suspension culture of *Arachis hypogaea*. As reported by Pamplin and Chapman [36], the formation of chlorophyll in *Daucus carota* cells was suppressed when the cells were grown on a sucrose-containing medium. Light and $CO_2$ are the growth-limiting factors for photoautotrophic plant cells, and favorable conditions concerning these factors have been demonstrated in suspension cultures of *A. hypogaea* [35], *Chenopodium rubrum* [37], and *Glycine max* [21, 38].

Thus far, most photoautotrophic plant cells have been induced in cultures of calli or suspension cells [21, 35–38]. Concerning plant hairy roots, Flores et al. [34] obtained the cell lines of photoautotrophic hairy roots of *A. oppositifolia* and *D. innoxia* through acclimation cultures with changes in sugar concentration in the medium and $CO_2$ tension in gas phase. However, detailed examinations have not been made to clarify the effects of culture conditions on the growth and photosynthetic activity of those photoautotrophic hairy roots.

### 3.3 Histological and Physiological Properties of Photoauto-, Photomixo-, and Hetero-trophic Hairy Roots

Histological examination was conducted to elucidate cellular changes in photoautotrophic and photomixotrophic green hairy roots stimulated by light. The Chl pigment [39] was found in the cortical cells of photoautotrophic hairy roots observed under a fluorescence microscope as compared with those of the photomixotroph. The heterotrophic hairy roots did not display the Chl pigment (data not shown).

Figure 4 shows a typical ultrastructure of hairy root cells observed under an electron microscope. In the case of photomixotrophic hairy roots, a plastid with a chloroplast-like structure was observed in the light-grown root cells, being associated with thylakoid membranes and grana stacks; the particles in the vi-

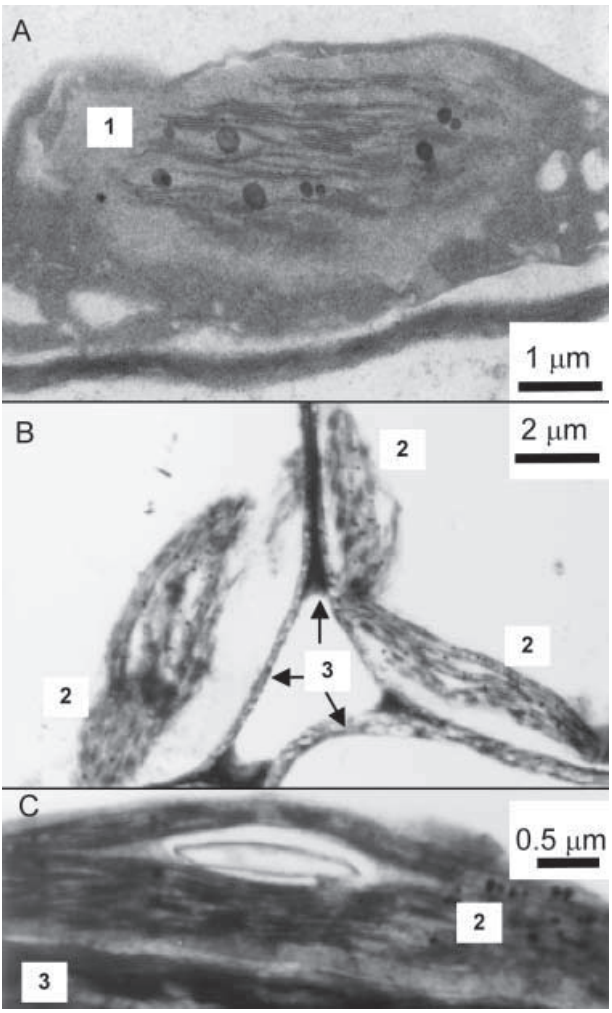


**Fig. 4A–C.** Electron micrographs of thin sections of pak-bung hairy roots: **A** photomixotrophic hairy roots obtained from a 13 days culture in light at $I = 11.1$ W m$^{-2}$); **B, C** photoautotrophic hairy roots cultivated in the sucrose-free medium with 3.0% $CO_2$-enriched air supply for 30 days using conical flasks illuminated at $I = 11$ W m$^{-2}$ and shaken at 100 rpm. The abbreviations of 1–3 indicates a chloroplast-like structure with thylakoid membranes and grana stacks (1), chloroplasts (2) and cell walls (3), respectively

cinity of the membranes were thought to be starch granules. Thus, plastids in green hairy root cells were endowed with a partially developed membrane system by exposure to light as observed in chloroplasts. Moreover, in the case of photoautotrophic hairy roots, electron microscopic examination revealed that the chloroplasts with a distinct membrane system were formed in the cells of the photoautotrophic hairy roots of pak-bung.

Flores and co-workers [7, 34] reported that *D. stramonium* green hairy roots exhibited enhanced production of alkaloids, especially scopolamine which is commonly found in field-grown plant leaves. Sauerwein et al. [8] demonstrated that *L. dulcis* green hairy roots accumulated significant amounts of terpenes, especially hernandulcin, and suggested that the terpenes were formed in connection with the development of chloroplasts in the root cells. These findings indicate the possibility that green hairy roots possess, at least in part, metabolic activities which function in parent plant leaves and stems, and that the altered productivities of metabolites can be obtained from the green hairy root cultures.

Table 3 compares the values of $C_{Chl}$, apparent $CO_2$ fixation rate, RubisCO activity, and some other enzyme activities among the hairy roots of different types and different parts of the parent plants of pak-bung. The $C_{Chl}$ and $A_R$ values in the hairy roots cultivated photomixotrophically with 30 kg $m^{-3}$ sucrose for 14 days were somewhat lower than those found in parent plant stems. Those values in the photoautotrophs were in excess of the values in the stems although even lower than those in parent plant leaves. Likewise, the $CO_2$ fixation rate of photoautotrophic hairy roots reached more than 3.0 times that of photomixotrophic ones.

Both APx and GPx are enzymes that scavenge hydrogen peroxide with ascorbate and guaiacoal as electron donors, respectively. It is known that APx is localized predominantly in chloroplasts and functions to eliminate toxic oxidant species generated by photochemical reactions in cooperation with superoxide dismutase [23]. Indeed, the activity of APx, $A_A$, in the leaves of parent plants was high compared with the less chlorophyllous organs of roots and stems as shown in Table 3. With regard to the APx activities of pak-bung hairy roots, the photoautotrophic hairy roots exhibited appreciably enhanced activity, as compared with those in the heterotrophic and photomixotrophic hairy roots. The relatively high activity of APx in the photoautotrophic hairy roots was consistent with the findings that the hairy roots possessed high Chl content

**Table 3.** Comparisons of the average Chl contents, apparent $CO_2$ fixation rates and some enzyme activities among the hairy roots of different types and different parts of the parent plants of pak-bung

| Plant materials | $C_{Chl}$ [g (kg-DW)$^{-1}$] | Apparent $CO_2$ fixation rate [mol-$CO_2$ (h · kg-DW)$^{-1}$] | $A_R$ [mol-$CO_2$ (h · kg-DW)$^{-1}$] | $A_A$ [U (kg-DW)$^{-1}$] | $A_G$ [U (kg-DW)$^{-1}$] |
|---|---|---|---|---|---|
| *Parent plants* | | | | | |
| Leaves | 16.3 | ND | 0.98 | 520 | 11 |
| Stems | 2.6 | ND | 0.15 | 201 | 14 |
| Roots | nil | ND | 0.02 | 27.0 | 53 |
| *Hairy roots* | | | | | |
| Heterotroph | nil | nil | 0.02 | 39.0 | 55 |
| Photomixotroph | 1.2 | $4.7 \times 10^{-2}$ | 0.12 | 132 | 75 |
| Photoautotroph | 5.6 | 0.15 | 0.26 | 384 | 80 |

ND: not determined.

and well-developed chloroplasts. Concerning GPx, it is known that this enzyme mainly exists in the cytosol of cells in plant roots [23]. Accordingly, as seen in Table 3, the relatively high value of GPx activity, $A_G$, was found in the roots of the parent plant in the present study. Moreover, the levels of $A_G$ activities detected in the cells of the three types of the hairy roots were comparable to those in the parent plant roots. It was thus found that photoautotrophic hairy roots contained both the APx and GPx enzymes functioning in the leaf and root organs, respectively.

Furthermore, the effect of $CO_2$ concentration on the photoautotrophic growth of pak-bung hairy roots was investigated in the range of $CO_2$ concentration in gas phase (0.03 – 8.5 % $CO_2$). The hairy roots cultivated under 0.03 % and 1.0 % $CO_2$ could not elongate at all for examined culture period of 14 days. The elongation rate of root tips increased with increasing $CO_2$ concentration up to 5.0 % and approached a saturated value. Flores et al. [34] found that supply with $CO_2$-enriched air (2.0 % $CO_2$) was necessary for the active growth of photoautotrophic hairy roots of *A. oppositifolia*. Rogers et al. [38] reported that suspension cells of *G. max* acclimated to photoautotrophy under 5 % $CO_2$ could not grow with a supply of plain air (0.03 % $CO_2$), and that RubisCO activity of the cells was lower than that of original plant leaves. In the present study, pak-bung hairy roots cultivated photoautotrophically also showed the low $A_R$ values ($A_R \cong 0.26$ mol-$CO_2$ $(h \cdot kg\text{-}DW)^{-1}$) as compared with that in parent plant leaves ($A_R \cong 0.98$ mol- $CO_2$ $(h \cdot kg\text{-}DW)^{-1}$).

Based on these results, photoautotrophic hairy roots of pak-bung were cultivated while being supplied with air containing 5.0 % $CO_2$ in subsequent studies.

### 3.4
### Influence of Photosynthetic Inhibitor on Elongation of Photoautotrophic Hairy Roots

For the investigation of influence of photosynthetic inhibitor on growth, the effect of DCMU concentration on the elongation rate, $R_G$, was examined as shown in Fig. 5. The elongation of heterotrophic hairy roots of pak-bung, which were cultivated on the medium containing 20 kg $m^{-3}$ sucrose in the dark, was not inhibited by the addition of 10 mmol $m^{-3}$ DCMU. In the photoautotrophic cultures of the hairy roots, the $R_G$ values decreased with increasing DCMU concentrations in the range 0 – 1.0 mmol $m^{-3}$, and the elongation was completely inhibited at DCMU concentrations of more than 1.0 mmol $m^{-3}$. Moreover, throughout the light intensities examined in the present study ($I = 2.6 – 32$ W $m^{-2}$), the $R_G$ values were zero in the media containing 10 mmol $m^{-3}$ DCMU (data not shown), indicating that the photoautotrophic growth of the hairy roots was completely suppressed at this level of DCMU.

These suggest that the proliferation of the derived cell line of photoautotrophic pak-bung hairy roots is dependent entirely on photosynthesis for acquiring carbon and energy sources because DCMU blocks the reaction in photosynthetic process as shown in Fig. 6. In addition, based on the sensitive response to DCMU, a kind of photosynthesis inhibitor, the photoautotrophic hairy roots may be used to detect some herbicides existing in the surroundings.

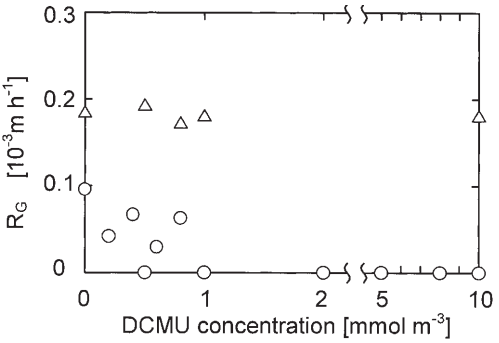


**Fig. 5.** Influence of DCMU concentration on elongation rate of growing points of photoautotrophic pak-bung hairy roots The hairy roots were cultivated in the sucrose-free medium using Petri dishes illuminated at $I = 11$ W $m^{-2}$. ○: photoautotrophic hairy roots, Δ: heterotrophic hairy roots

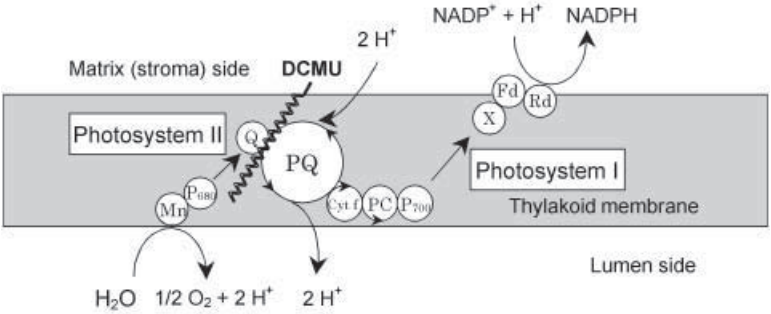


**Fig. 6.** Sites of inhibitory action of DCMU in photosynthetic electron transport chain. The abbreviations are as follows – Cyt f: cytochrome f, Fd: ferredoxin, Mn: water-splitting complex (manganese-containing), P680: pigment complex of photosystem II, P700: pigment complex of photosystem I, PC: plastocyanin, PQ: plastquinone, Q: quencher, Rd: NADP reductase and X: direct electron acceptor complex

Table 4 summarizes properties of various plant cells with photosynthetic ability, indicating the Chl contents, RubisCO activities, and apparent $CO_2$ fixation rates of photoautotrophic cells derived from various plants [34, 38, 40]. The callus of *G. max* possessed the Chl content of $1.4 - 1.9 \times 10^3$ mg (kg-FW)$^{-1}$, the value of which was about 28-fold over that in the callus of *N. tabacum* (58 mg (kg-FW)$^{-1}$). The photoautotrophic hairy roots of pak-bung exhibited relatively high Chl content of $6.1 \times 10^2$ mg (kg-FW)$^{-1}$ though the value was lower than that of *G. max* callus. On the other hand, as shown in Table 4, the levels of RubisCO activity and Chl-based $CO_2$ fixation rate of the pak-bung hairy roots were comparable to those of the photoautotrophic plant cells reported in the literatures. These values, therefore, can be used as indicators for the potentials of photoautotrophic growth of plant cells.

**Table 4.** Chl contents, RubisCO activities and apparent $CO_2$ fixation rates of photoautotrophic cells derived from various plants

| Cells | $CO_2$ and light conditions | Chl content [mg (kg-FW)$^{-1}$] | RubisCO activity [μmol-$CO_2$ (h · mg-Chl)$^{-1}$] | Apparent $CO_2$ fixation rate [μmol-$CO_2$ (h · mg-Chl)$^{-1}$] | Ref. |
|---|---|---|---|---|---|
| *N. tabacum* var. Samsun callus | 1% $CO_2$, 100, 000 lux | 58 | 54 | ND | 40 |
| *Cytisus scoparius* Link callus | 1% $CO_2$, 100, 000 lux | 133 | 53 | ND | 40 |
| *Phellodendron amurense* Rupr. callus | 1% $CO_2$, 100, 000 lux | ND | 57 | ND | 40 |
| *G. max* L. Merr. var. Corsoy callus | 5% $CO_2$, 250–300 μE/m$^2$/s | 1.4 – 1.9 × $10^3$ | 64 – 91 | 35 – 61 | 38 |
| *A. oppositifolia* Hairy roots | 2% $CO_2$, 70 $\mu$ E/s m$^2$ | 178.6 | 54.5 | 31.3 | 34 |
| *I. aquatica* hairy roots | 5% $CO_2$, 11 W m$^{-2}$ | 6.1 × $10^2$ | 47 | 27 | 15 |

The following relationship describes the conversion to light intensity units: 1000 lux ≅ 13 μE (s/m$^2$)$^{-1}$ ≅ 3.4 W m$^{-2}$.
ND: not determined.

# 4
# Kinetic Analysis of Hairy Root Cultures under Light Irradiation

## 4.1
## Kinetic Expression of Growth and Chlorophyll Formation of Hairy Roots

Achieving the efficient production of desired metabolite requires knowledge of how to find the key factor to enhance the metabolite productivity as well as how to describe kinetics considering the effect of the factor. However, there are few reports concerned with kinetic expression of the metabolite production in the hairy root culture [41, 42]. For the broad application of hairy roots to the metabolite production, therefore, various types of kinetic expressions should be proposed.

To evaluate the growth and Chl formation of pak-bung hairy roots, a kinetic model for the growth by root elongation and ramification at growing point is presented.

### 4.1.1
### *Modeling of Hairy Root Growth*

According to the morphological properties of branching and elongation, the following assumptions are made for the modeling of hairy root growth as illustrated in Fig. 7 [43]:

1. The hairy roots grow in a manner of one-dimensional extension at root tip meristem (growing point, GP) with length $L_G$.
2. The binary division of GP occurs within negligible time on hairy root branching. Each GP grows and forms "highly branched hairy root".
3. Environmental factors such as shear stress cause GP decay.
4. The root is regarded as a cylinder with diameter $D$ and length $L$.

On the basis of assumption 1, a linear growth law for one GP is introduced:

$$dL/dt = \mu \cdot L_G \tag{3}$$

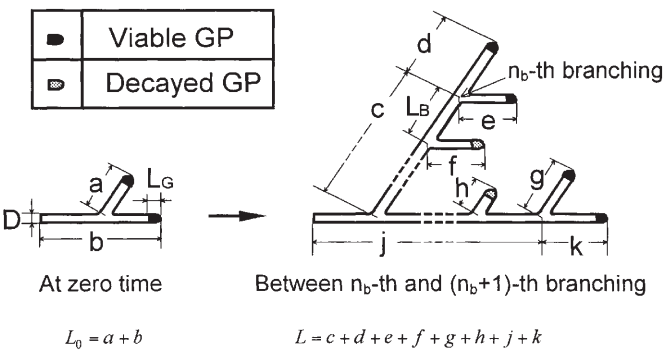


**Fig. 7.** Schematic drawing of hairy root branching growth

where $\mu$ is the specific elongation rate of GP. The time until next branching (division) of GP, $\Delta t$, is given by

$$\Delta t = \int_0^{L_B} dL/(\mu L_G) \tag{4}$$

Between the $n_b$-th and $(n_b+1)$-th branching of GP ($t_{I,n_b} \le t < t_{I,n_b+1}$), the initial conditions are

$$t = 0;\ L = L_0,\ N = N_0\ (n_b = 1) \tag{5}$$

$$t = t_{I,n_b};\ L = t_{I,n_b},\ N = N_{I,n_b}\ (n_b \ge 2) \tag{6}$$

where $N$ shows the number of GPs and the subscripts 0, $I$ and $n_b$ denote zero time, initial time for $n_b$-th branching and $n_b$-th branching of GP, respectively.

The variation in viable GPs between the $n_b$-th and $(n_b+1)$-th branching is expressed as follows;

$$\begin{array}{ll} t_{I,n_b} \le t < \omega_1; & N = N_{I,n_b} \\ \omega_1 \le t < \omega_2; & N = N_{I,n_b} - 1 \\ \vdots\!\vdots & \\ \omega_m \le t < t_{I,n_b}; & N = N_{F,n_b} - m \\ i = 0, 1, 2, \cdots, m & \end{array} \tag{7}$$

where $m$ shows the number of decayed GPs between the $n_b$-th and $(n_b+1)$-th branching.

Here, the decay rate of GPs is expressed as follows.

$$dN/dt = -k_d N \tag{8}$$

Thus, $i$ of GPs decay at $t = \omega_1$,

$$\omega_i = (1/k_d) \ln \{N_{I,n_b} - i)\} + t_{I,n_b} \tag{9}$$

At the $(n_b+1)$-th branching, the initial number of GPs, $N_{I,n_b+1}$, are calculated as follows.

$$N_{I,n_b+1} = 2\, N_{F,n_b} \tag{10}$$

Thus, overall root length, $L$, can be obtained by integrating Eq. (3) with respect to $N$ of GPs.

The concentration of dry cell mass, $X$, can be calculated from the following equation:

$$X = \rho\pi (1 - W_C)\, LD^2/4V \tag{11}$$

### 4.1.2 *Modeling of Chlorophyll Formation*

The kinetic expression for the Chl formation was made according to a pigment formation model proposed in the previous paper [42]. In this model, it was postulated that the pigment was accumulated in an organelle by means of per-

meation of pigment or its precursor through an envelope from cytoplasm into organelle. For the Chl formation, pak-bung hairy roots under light irradiation was postulated to accumulate the Chl inside chloroplast through its envelope. Figure 8 illustrates the conceptual drawing of single hairy root with distribution of Chl content. A GP ($L_G$ in length) is postulated to exist at the tip part of a cylindrical single hairy root ($D$ in diameter). One hairy root is divided into two sections; growth section within GP ($0 \le l \le L_G$) where the root elongation occurs through cell division, and Chl formation section except GP ($L_G < l \le L$) where no cell division occurs and Chl is synthesized via the secondary metabolism in each cell. Particular cells existing in the range from $l$ to $l + dl$ have their own cellular ages of $\theta$ to $\theta + d\theta$, being matured with increasing value of $l$. A gradient of Chl content, $\hat{C}_{Chl}(t, l)$, consequently, appears depending on cellular age along a hairy root.

From the assumption described above, the rate of the Chl formation at a given distance from tip, $l$, of a single hairy root is expressed as follows.

$$\partial \hat{C}^{i}(t, l)/\partial l = \begin{cases} 0 & (0 \le l \le L_G) \\ k_c(\hat{C}^{o} - \hat{C}^{i}(t, l)) & (L_G < l) \end{cases} \qquad \text{(12 and 13)}$$

where $k$ is apparent permeation rate constant through the envelope of chloroplast. In the case of the hairy roots in the dark, the value of $\hat{C}^{o}$ in Eq. (13) is postulated as zero if Chl in the chloroplast is not accumulated.

The overall amount of Chl in hairy roots, $M$, can be calculated by the integration of Eqs. (12) and (13) as described previously [42]:

$$M = \frac{\pi \alpha D^2}{4} \int_0^{L_B} \hat{C}^{i}(t, l)\, dl \qquad (14)$$

From experimental measurements, $\hat{C}^{i}(t, l)$ and $\hat{C}^{0}$ in Eq. (13) are expressed as $\hat{C}_{Chl}(t, l)$ and $\hat{C}_{Chl,S}$ by the following equations, respectively:

$$\hat{C}_{Chl}(t, l) = \frac{\alpha \hat{C}^{i}(t, l)}{\rho(1 - W_C)} \text{ and } \hat{C}_{Ch,S} = \frac{\alpha \hat{C}^{o}}{\rho(1 - W_C)} \qquad (15)$$

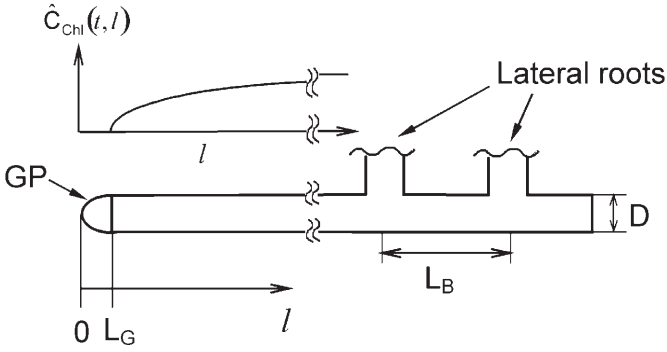


**Fig. 8.** Conceptual drawing of a single hairy root with distribution of chlorophyll content

The amount of Chl on a culture volume basis, $A_{Chl}$, and average content of chlorophyll in the hairy roots, $C_{Chl}$, are calculated as follows, respectively:

$$A_{Chl} = M/V \tag{16}$$

$$C_{Chl} = M/VX \tag{17}$$

### 4.2 Influence of Light Intensity on Photomixotrophic and Photoautotrophic Growth of Hairy Roots

In general, the growth of hairy roots occurs through the elongation and branching at growing points. Therefore, the elongation rate, $R_G$, and the number of growing points, $N$, determine the growth rate of hairy roots.

In the cases of the cultures in sugar-containing media, it was reported that the $R_G$ value is not greatly affected by light exposure [14]. The specific elongation rate, $\mu$, of heterotrophic and photomixotrophic hairy roots is expressed as a Monod-type equation where fructose is a limiting substrate:

$$\mu = \mu_{Fmax}\, C_F/(K_F + C_F) \tag{18}$$

The parameter values of $\mu_{Fmax}$ and $K_F$ were determined according to the procedure described by Taya et al. [43] as shown in Table 5 from separate cultures of pak-bung hairy roots.

With respect to the photoautotrophic hairy roots, the $R_G$ values sharply increased with increasing $I$ values and apparently approached a saturated value as shown in Fig. 9. The relationship between the values of $\mu$ and $I$ was expressed by the following equation where incident light intensity is a limiting factor:

$$\mu = \mu_{Imax}\, I/(K_I + I) \tag{19}$$

**Table 5.** Values of parameters and constants used for calculation

| Photoautotroph | Photomixotroph |
|---|---|
| $\hat{C}_{Chl,S} = 8.9$ g (kg-DW)$^{-1}$ | $\hat{C}_{Chl,S} = 0$ g (kg-DW)$^{-1}$ (under darkness), 2.5 g (kg-DW)$^{-1}$ (under light irradiation) |
| $K_I = 0.69$ W m$^{-2}$ | $K_F = 5.9$ kg m$^{-3}$ |
| $k_c = 0.58$ h$^{-1}$ | $k_c = 1.2 \times 10^{-2}$ h$^{-1}$ (2.5 g (kg-DW)$^{-1}$ (under light irradiation) |
| | $Y^* = 0.51$ |
| $\chi$ =4 .6 | $\chi = 1.3$ |
| $\sigma = 44$ | $\sigma = 2.3$ |
| $\mu_{Imax} = 0.20$ h$^{-1}$ | $\mu_{Fmax} = 0.49$ h$^{-1}$ |
| $\varepsilon = 2.7 \times 10^{-2}$ h$^{-1}$ | |
| $\phi = 12$ W m$^{-2}$ | |

Other values of constants and parameters in common with hairy roots [43].
$D = 1.0 \times 10^{-3}$ m, $L_{BD} = 1.5 \times 10^{-3}$ m, $L_G = 5.0 \times 10^{-4}$ m, $W_C = 0.85$, $\rho = 1.01 \times 10^3$ kg-FW m$^{-3}$.

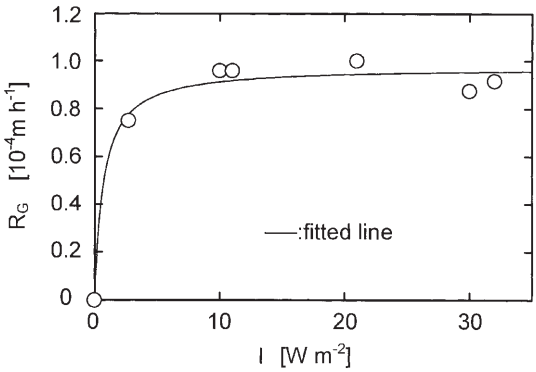


**Fig. 9.** Relationship between elongation rate of growing points and incident light intensity for photoautotrophic hairy roots

The constant values of $\mu_{Imax}$ and $K_I$ were determined as listed in Table 5 by fitting the experimental data shown in Fig. 9 using the non-linear least squares method.

In addition, with decreasing the interval between neighboring branches, $L_B$, the doubling time of growing points decreases. The root growth is enhanced since the high branching frequency results in the increase in the number of growing points, $N$. Figures 10 and 11 show the effect of light intensity on the $L_B$ values under photomixotrophic and photoautotrophic conditions, respectively. In both cases, ($L_{BD}/L_B$) values gradually increased with increasing $I$ values. To correlate $L_B$ with $I$, the following equation was employed:

$$L_{BD}/L_B = 1 + \chi I/(\sigma + I) \tag{20}$$

The parameters of $\chi$ and $\sigma$ were determined as 1.3 (photomixotroph) or 4.6 (photoautotroph) and 2.3 (photomixotroph) or 44 (photoautotroph) W $m^{-2}$,

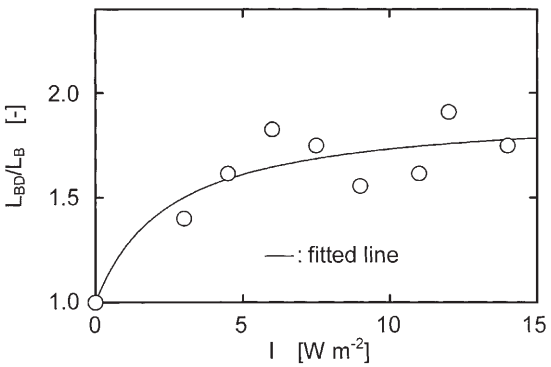


**Fig. 10.** Relationship between interval between neighboring branches and incident light intensity for the photomixotrophic hairy roots

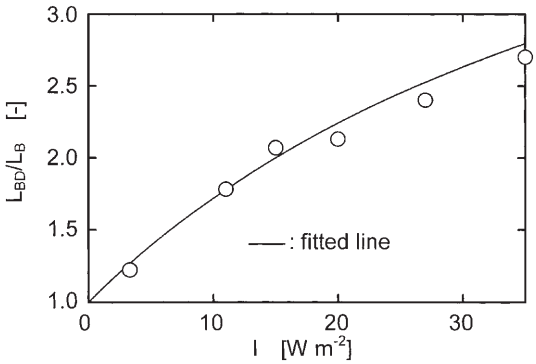


**Fig. 11.** Relationship between interval between neighboring branches and incident light intensity for the photoautotrophic hairy roots

respectively, by fitting the experimental data using the non-linear least squares method.

Repunte et al. [44] and Nakashimada et al. [45] reported that horseradish hairy roots exhibited the frequent ramification when the hairy roots were cultivated in the medium containing phytohormone, auxin such as 1-naphthaleneacetic acid. In the present study, therefore, the content of IAA, which is a kind of natural auxin, was measured in photomixotrophic pak-bung hairy roots culturd for 312 h with and without light irradiation ($I = 0$ and 11.1 W m$^{-2}$). IAA contents at $I = 0$ and 11.1 W m$^{-2}$ were 170 µg and 280 µg (kg-DW)$^{-1}$, respectively. It was suggested that light exposure of pak-bung hairy roots stimulated the IAA synthesis in the cells and thus the branch interval of roots was shortened, although the biological mechanism of promoted IAA formation in pak-bung green hairy roots under the light irradiation was not explicitly shown.

### 4.3
### Characterization of Chl Formation in the Hairy Root Cells Under Light Irradiation

For the estimation of Chl formation in hairy roots, the root cells were examined on a laser scanning confocal image system attached to a microscope. The filter package with a laser (excitation 568 nm) and a 590–610 nm band pass barrier filter for red fluorescence was employed. Digital image analysis was performed by a computer-aided image processing system with software.

The Chl pigment was localized in the cortical cells of the photoautotrophic and photomixotrophic hairy roots. Thus, the vertical sections of the cortical cells, i. e., a plane lying in the position with distance of 15 µm (photoautotroph) or 30 µm (photomixotroph) from the uppermost epidermis, was examined. Regarding each point as one chloroplast, the number of chloroplast per unit area of the vertical section, $N_{Chl}$, was determined.

The distribution of Chl content was observed in pak-bung hairy roots cultivated under light irradiation. To clarify the distribution, photoautotrophic and photomixotorophic hairy roots, which were cultivated for 672 h or 240 h, re-

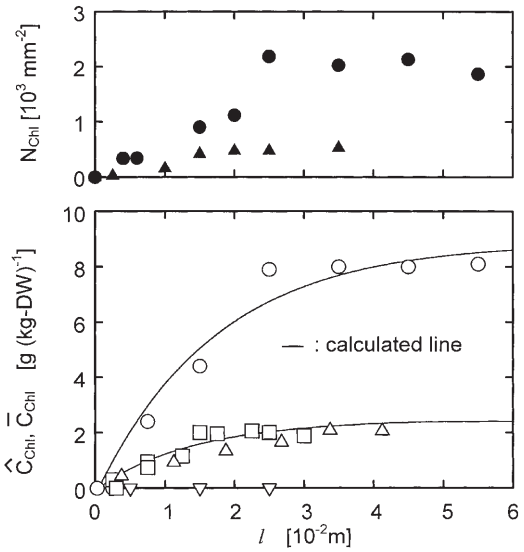


**Fig. 12.** Relationship of sectional Chl content in segments and number of chloroplasts per unit area of vertical sections of hairy roots against mean distance from root tip. ○: photoautotrophic culture at $I = 11$ W/m$^2$ for $t = 672$ h, □ :photomixotrophic culture at $I = 11$ W/m$^2$ for $t = 240$ h, △: photomixotrophic culture at $I = 1.8$ W/m$^2$ for $t = 240$ h, ▽ photomixotrophic culture at $I = 0$ W/m$^2$ for $t = 240$ h. Open and close symbols present the sectional Chl content in segment and number of chloroplast per area of vertical sections of hairy roots, respectively

spectively, were cut and divided into several segments. Figure 12 shows the relationships between sectional Chl contents in the segments, $\bar{C}_{Chl}(t, l)$, and mean distances from the tip of the hairy roots, $l$. $\bar{C}_{Chl}(t, l)$ values in the hairy roots cultured in the dark were approximately zero in the range of $l = 0 - 25 \times 10^{-3}$ m, indicating that Chl was not formed in hairy roots. In hairy roots exposed to light, $\bar{C}_{Chl}(t, l)$ values increased with increasing $l$ values. The $\bar{C}_{Chl}(t, l)$ values of photoautotrophic hairy roots were higher than those of photomixotrophic hairy roots in each segment examined in this study. No significant difference in $\bar{C}_{Chl}(t, l)$ values between $I = 1.8$ and 11 W m$^{-2}$ was observed in photomixotrophic hairy roots. To estimate $k_C$ and $\hat{C}_{Chl}(t, l)$ values in Eqs. (13) and (15), the calculated values of $\hat{C}_{Chl}(t, l)$ were fitted to the experimental ones using the non-linear least squares method. The calculated values indicated by the solid lines in Fig. 12 were obtained by integration of Eqs. (3), (12), and (13). The obtained values of $k_C$ and $\hat{C}_{Chl,S}$ are listed in Table 5. The $\hat{C}_{Chl,S}$ value at $I = 0$ W m$^{-2}$ was regarded as zero.

For detailed investigation of the distribution of Chl content, the vertical sections of the photoautotrophic and photomixotrophic hairy roots at various distances from the root tips were observed under laser scanning confocal microscopy. The density of chloroplasts, $N_{Chl}$, in the cortical cells of the hairy roots was estimated. Figure 12 illustrates the longitudinal distribution of the $N_{Chl}$ values in the photoautotrophic and photomixotrophic hairy roots. The $N_{Chl}$ values in-

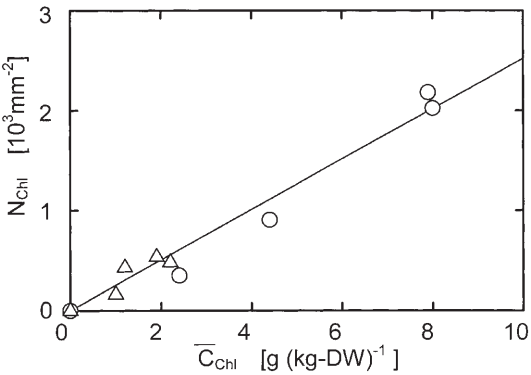


**Fig. 13.** Relationship between Chl content and the number of chloroplasts per unit area of vertical section of hairy roots. ○ photoautotrophic hairy roots, △: photomixotrophic hairy roots

creased with increasing $l$ values in analogy with the $\overline{C}_{Chl}$ values in regard to both the photoautotrophic and photomixotrophic hairy roots. The $N_{Chl}$ value was directly proportional to the $\overline{C}_{Chl}$ value as shown in Fig. 13. It was thus found that the distribution of $\overline{C}_{Chl}$ values in the hairy roots could be interpreted by changes of $N_{Chl}$ values in the cells. In addition, it was suggested that the image analysis method described here is a useful tool to determine the topical Chl content.

A longitudinal distribution of product content along the roots was also recognized in red beet hairy roots which produced the pigment betanin [42]. The distribution of product content in a longitudinal direction was considered to be attributed to cellular age distribution arising from the linear growth mode of the roots. Concerning Chl, time for development of chloroplast, a kind of cell organelles, might affect the distribution of the Chl content. In the following section, the kinetic analysis of Chl formation in the photomixotrophic hairy roots is carried out regarding the Chl as a secondary metabolite.

### 4.4
### Profiles of Photoauto- and Photomixo-trophic Cultures of Hairy Roots under Light

Photomixotrophic and photoautotrophic cultures of pak-bung hairy roots were performed at various incident light intensities.

Figure 14 shows the time courses of the cultures with and without light exposure ($I = 0$ and 11 W m$^{-2}$) using the photomixotrophic hairy roots. At light intensity of $I = 11$ W m$^{-2}$, active growth was observed and the root mass concentration, $X$, reached 9.9 kg-DW m$^{-3}$ at $t = 447$ h.

After 447 h, the $X$ value did not increase due to the depletion of fructose in the medium. Furthermore, the average content of Chl, $C_{Chl}$, in the hairy roots and the amount of Chl, $A_{Chl}$, in the culture flask increased with progressing $t$ value. On the contrary, in the culture without light irradiation ($I = 0$ W m$^{-2}$), the $C_{Chl}$ value decreased and was close to zero after $t = 360$ h.

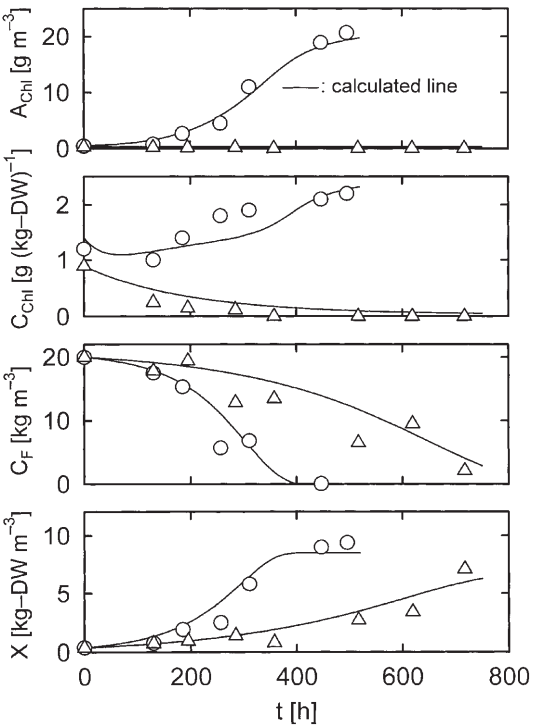


**Fig. 14.** Time courses of cultures of photomixotrophic hairy roots at $I = 0$ (△) and 11 W/m$^2$ (○), respectively

To verify the kinetic model described by Eqs. (3) – (20), the time profiles of root growth, fructose consumption, and Chl formation were calculated in photomixotrophic cultures. Here, fructose concentration in the medium, $C_F$, was calculated based on a rate equation including the true cell mass yield, $Y^*$, and maintenance energy, $m_S$, as described elsewhere [46]. The values of $Y^*$ and $m_S$ were estimated as shown in Table 5 from separate cultures of pak-bung hairy roots. The calculation was carried out using the initial values of $X = 0.4$ kg-DW m$^{-3}$, $C_F = 20$ kg m$^{-3}$, and $C_{Chl} = 1.0 \times 10^{-3}$ kg (kg-DW)$^{-1}$, and the constant and parameter values listed in Table 5. The calculated results for $X$, $C_F$, $C_{Chl}$, and $A_C$, which are represented by the solid lines in Fig. 14, agreed closely with the experimental values. In addition, comparisons between experimental data and calculated values for $X$, $C_F$, $C_{Chl}$, and $A_C$ of the hairy roots cultivated at various light intensities for 240 h are shown in Fig. 15. The calculated values fairly coincided with the experimental data. The kinetic model presented in this study, thus, could be valid to express the behaviors of the growth and Chl formation in the cultures of photomixotrophic hairy roots with and without light irradiation.

In the case of photoautotrophic cultures of pak-bung hairy roots at light intensities of $I = 0 - 31$ W m$^{-2}$, as shown in Fig. 16, as expected, no growth of the hairy roots was observed in the dark. $X$ values increased with increasing $I$

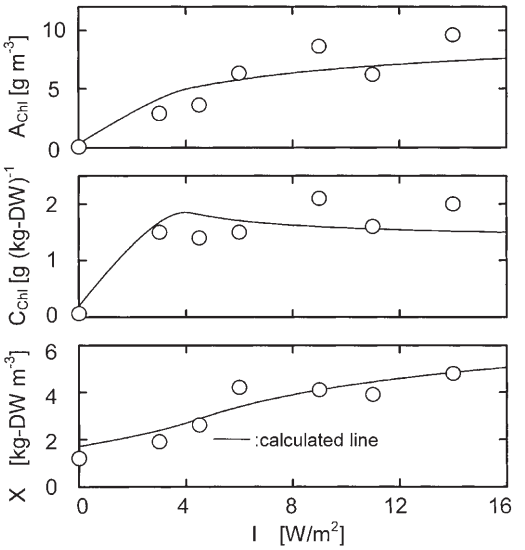


**Fig. 15.** Relationships between incident light intensity and root mass concentration, average Chl content and Chl amount on a culture volume basis in photomixotrophic cultures of hairy roots at 240 h

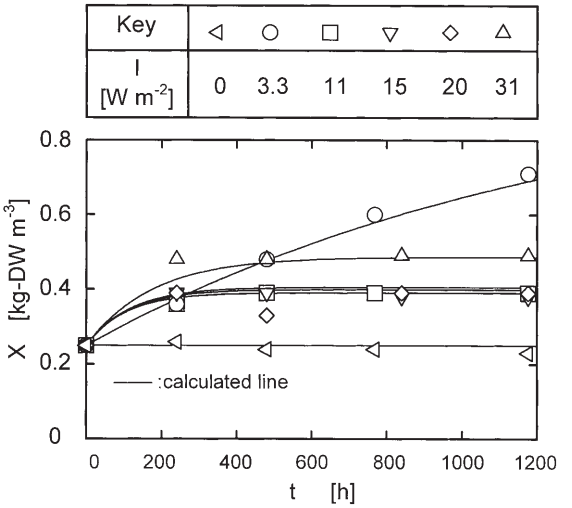


**Fig. 16.** Time courses of cultures of photoautotrophic hairy roots at various incident light intensities

values at culture time of $t = 240$ h, while the notable growth of roots was obtained afterwards only in the culture at $I = 3.3$ W m$^{-2}$. At light intensity of $I = 3.3$ W m$^{-2}$, $X$ value reached 0.71 kg-DW m$^{-3}$ at $t = 1176$ h.

However, photoautotrophic growths of the hairy roots at various light intensities could not be expressed satisfactorily by Eqs. (3)–(20). It is well known that exposure to strong light causes damage to the photosynthetic cells. The photoautotrophic cell line of pak-bung hairy roots possessed high photosynthetic potential and was dependent entirely on photosynthesis for acquiring their carbon and energy sources. To investigate the harmful effect of light, in the present study, the influence of light intensity on the viability of growing points of photoautotrophic hairy roots was examined. Figure 17 shows the time courses of the values of $(-\ln (N/N_I))$ under various incident light intensities ($I = 4.5 - 27$ W m$^{-2}$). Throughout light intensities examined, the values of $(-\ln (N/N_I))$ increased linearly with elapsed time culture.

According to Eq. (8), the decay rate of growing points is given by the equation

$$-\ln (N/N_I) = k_D\, t \tag{21}$$

where $N_I$ is the initial value of $N$. Therefore, the slopes obtained from the plots of $(-\ln (N/N_I))$ vs $t$ give the value of $k_D$.

As shown in Fig. 18, it was found that $k_D$ increased with increasing value of $I$. To correlate $k_D$ with $I$, the following equation was employed:

$$k_D = \varepsilon\, I/(\phi + I) \tag{22}$$

The parameters of $\varepsilon$ and $\phi$ were determined as $2.7 \times 10^{-2}$ h$^{-1}$ and 12 W m$^{-2}$, respectively, by applying Eq. (22) to experimental data using the non-linear squares method.

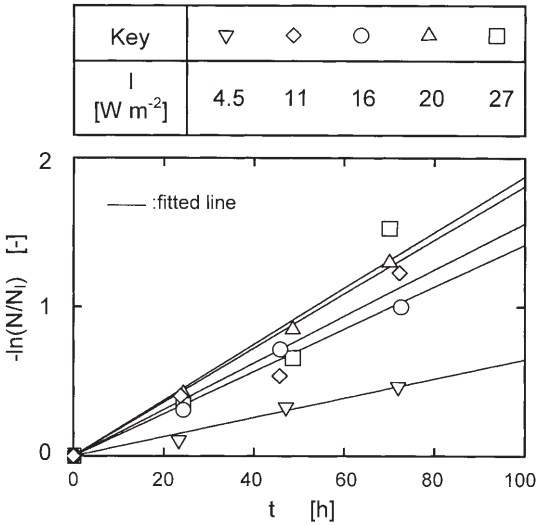


**Fig. 17.** Time profiles of the number of viable growing points at various incident light intensities in photoautotrophic hairy root cultures

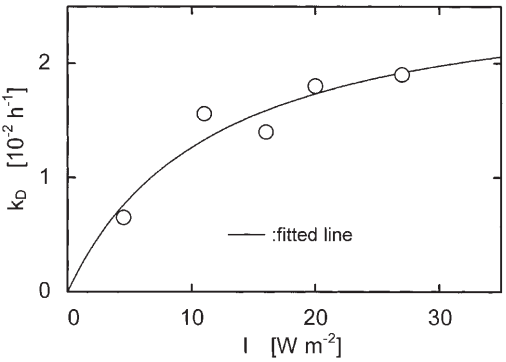


**Fig. 18.** Relationship between decay rate constant and incident light intensity for photoautotrophic hairy roots

It was found that strong light exerted a harmful effect on the hairy roots although it was necessary to support the photoautotrophic growth of the hairy roots. Fischer and Alfermann [37] reported that when photoautotrophic cells of *C. rubrum* were cultivated under strong light irradiation, cell growth rate was reduced during the early culture period accompanied with marked reduction in Chl content. Hirayama et al. [47] demonstrated that in the cells of *Chlorella valgaris*, the hydroxyl radical content increased and the photosynthetic efficiency decreased with increasing light intensity. In our previous studies, kinetic growth expressions were presented to formulate the photo-inhibition phenomena in the cultures of *Spirulina platensis* and *Marchantia paleacea* [48, 49].

It was thus considered that toxic oxidant species such as $O_2^{\bullet-}$ and $H_2O_2$ produced by photochemical reactions in the cells may be attributable to the decay of growing points of pak-bug hairy roots.

The time courses of the photoautotrophic hairy root cultures at various light intensities were calculated based on the model taking account of the relationship between decay rate of growing points and light intensity expressed by Eq. (22). The calculated values agreed with experimental data at each $I$ value as represented by the solid lines in Fig. 16. Figure 19 shows the relationships between the values of $X$ and $I$ at culture time of $t = 240$ and 1176 h. The number of active growing points might decrease with time at relatively high light intensities due to high $k_D$ values as indicated in Fig. 17. For extended cultivations, therefore, it was considered that the photoautotrophic hairy roots exhibited a satisfactory growth at the moderate light intensity such as $I = 3.3$ W $m^{-2}$.

### 4.5
### Relationship Between Longitudinal Distribution of Chlorophyll Content and Available Energy in Hairy Root Cells Under Photoautotrophic Condition

Figure 20 shows the relationship between the values of root length, $L$, and initial elongation rate of growing points, $R_{GI}$, in the photoautotrophic cultures of pak-bung hairy roots. The $R_{GI}$ values increased with increasing $L$ values and appa-

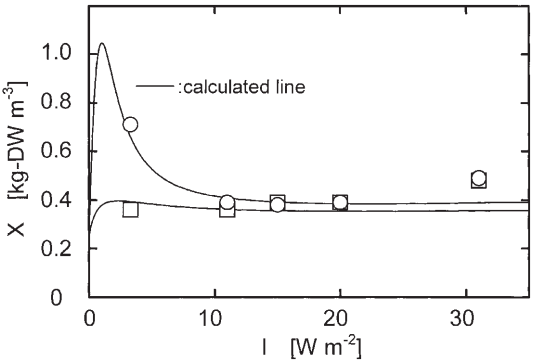


**Fig. 19.** Relationships between root mass concentration and incident light intensity in photoautotrophic cultures of hairy roots at 240 h (□) and 1176 h (○)

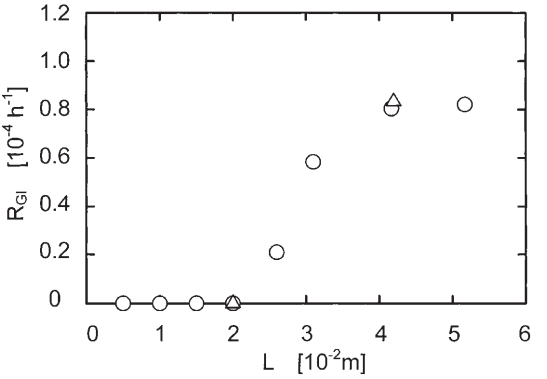


**Fig. 20.** Relationship between initial elongation rate of growing points and root length at I = 11 (○) and 20 W $m^{-2}$ (△)

rently approached a saturated value. The photoautotrophic hairy roots of pak-bung without the cutting treatment described above exhibited the $R_G$ value of $9.6 \times 10^{-5}$ m $h^{-1}$ at light intensity of $I = 11$ W $m^{-2}$ (Fig. 9) and this value was equal to the $R_{GI}$ value at $L = 9.1 \times 10^{-2}$ m under $I = 11$ W $m^{-2}$. Thus, the saturated $R_{GI}$ value was estimated to be $9.6 \times 10^{-5}$ m $h^{-1}$ for the hairy roots with sufficient length. No significant difference was observed in the $R_{GI}$ values between light intensities of $I = 11$ and 20 W $m^{-2}$. It was therefore indicated that the incident light intensity was not a limiting factor for the elongation rate under the examined condition. On the other hand, it was noteworthy that the threshold of $R_{GI}$ value was found concerning the $L$ value, that is, no elongation was observed when the $L$ values were less than $2.0 \times 10^{-2}$ m.

Similar phenomena were observed in the heterotrophic cultures of pak-bung and tobacco hairy roots with respect to sucrose concentration in the medium [50]. That is, no elongation occurred when the hairy roots were cultivated on the medium with sucrose concentrations of $C_{SI} = 0 - 2.5$ kg $m^{-3}$. It was suggested

that the hairy root cells can elongate merely when they capture the sufficient amount of sugar as a source of energy to maintain their viability. Under the lowered sugar concentration, the supplied sugar does not meet the synthesis of cell mass and the hairy root cells enter a dormancy where the energy is mainly consumed for maintenance metabolisms.

The photoautotrophic cells gain energy and carbon entirely via photosynthetic reactions. It is known that the content of Chl greatly affects photosynthetic potential of plant cells. Figure 21 indicates the calculation of relationship between the average Chl content, $C_{Chl}$, of a single root and its length, $L$. As described in the previous section, the longitudinal distribution of Chl content, $\bar{C}_{Chl}$, along the roots (Fig. 12), which was expressed by Eqs. (12) and (13), was recognized in the photoautotrophic hairy roots of pak-bung. The $\bar{C}_{Chl}$ values increased with increasing $l$ values. Therefore, the calculated value of $C_{Chl}$ by Eq. (17) increased with $L$ value and it was suggested that the photosynthetic potential of a single root would increase with increasing length of a single hairy root, resulting in the enhancement of the $R_{GI}$ value.

To correlate the Chl content and the photosynthetic potential of the hairy roots in detail, RubisCO activity was investigated because it is a key enzyme of $CO_2$ fixation in photosynthetic reactions. Figure 22 shows the relationship between the average RubisCO activity, $A_R$, and $C_{Chl}$ of pak-bung hairy roots cultivated under various conditions. The $A_R$ value was directly proportional to the $C_{Chl}$ value in pak-bung hairy roots and correlated by the following equation:

$$A_R = \gamma C_{Chl} \tag{23}$$

The value of $\gamma$ was determined as $4.7 \times 10^{-2}$ mol-$CO_2$ $(h \cdot g)^{-1}$ by fitting Eq. (23) to the experimental data using the least squares method.

Provided that the average apparent $CO_2$ fixation rate is directly proportional to the $A_R$ value, the following equation can be obtained:

$$v_{CF} = \eta A_R \tag{24}$$

The $\eta$ value was estimated as 0.57 from the data shown in Table 4.

It is assumed that the reaction catalyzed by RubisCO is a rate-limiting step in reactions of photosynthetic carbon fixation expressed as follows in the lump:

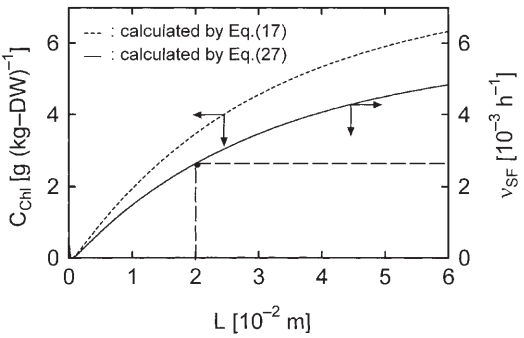


**Fig. 21.** Relationship of average Chl content and average sucrose formation rate per dry cell weight against length of a single root

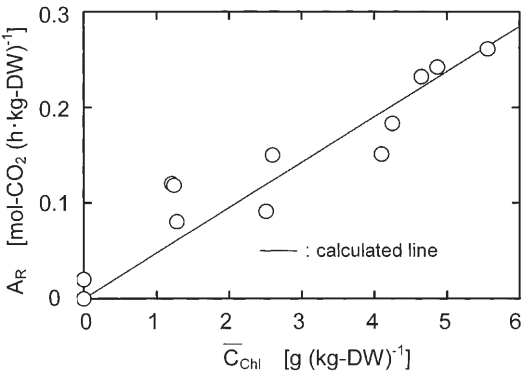


**Fig. 22.** Relationship between average Chl content and average RubisCO activity in hairy roots cultivated under various conditions

$$12CO_2 + 11H_2O \rightarrow C_{12}H_{22}O_{11} + 12O_2 \tag{25}$$

Therefore, average carbon fixation rate per dry cell weight based on sucrose, $v_{SF}$, is given as follows:

$$v_{SF} = \iota\, v_{CF} \tag{26}$$

The $\iota$ value was determined as $2.8 \times 10^{-2}$ kg mol$^{-1}$ from the stoichiometric relationship.

Combination of Eqs. (24) – (26) and Eq. (13) yields the following equation to correlate the $v_{SF}$ value with the $L$ value:

$$v_{SF} = \Theta \int_0^L C_C(t, l)\mathrm{d}l/\mathrm{L} \tag{27}$$

where $\Theta = \beta\, \gamma\, \eta\, \iota$.

The value of $v_{SF}$ is regarded as energy captured as an organic compound by the hairy root cells per unit cell mass per time. This value, thus, could be utilized to discuss the growth potential of the hairy root cells.

Figure 21 shows the $v_{SF}$ value of a single root with the length of $L$ calculated by Eq. (27). The $v_{SF}$ values increased with increasing $L$ values. The value of $v_{SF}$ at the root length of $L = 2.0 \times 10^{-2}$ m, at which the threshold of elongation was recognized, was calculated as $2.7 \times 10^{-3}$ h$^{-1}$. The value was of the same order as the values of maintenance energy for pak-bung hairy root cells based on sugar consumption rate ($m_S = 5.0 \times 10^{-3}$ h$^{-1}$) or $O_2$ uptake rate ($m_R = 3.1 \times 10^{-3}$ h$^{-1}$) determined in the previous section.

It was suggested that the similar phenomenon observed in the hairy root cells under conditions of limited energy supply could be interpreted in terms of energy captured by the cells and maintenance energy. Based on these findings, critical energy required by the pak-bung hairy root cells to maintain their viability was estimated to be around $3.0 \times 10^{-3}$ h$^{-1}$ as sucrose equivalent.

## 5 Concluding Remarks

The hairy roots of pak-bung turned green when cultured under continuous light exposure, and maintained their branched root morphology with the formation of chlorophyll. The green photomixotrophic hairy roots cultivated under light exhibited increased root growth and enhanced activities of superoxide dismutase, SOD, and peroxidase, POD. A light intensity of 11.1 W $m^{-2}$ using white fluorescent lamps was found to support good root proliferation and relatively high activities of SOD and POD. In cultures of green hairy roots at a light intensity of 11.1 W $m^{-2}$, root growth, total SOD activity, and total POD activity obtained on culture day 21 were 8.1 kg-DW $m^{-3}$, $18 \times 10^7$ U $m^{-3}$, and $40 \times 10^6$ U $m^{-3}$, respectively. In dark culture, the corresponding values obtained on culture day 21 were 3.8 kg-DW $m^{-3}$, $3.0 \times 10^7$ U $m^{-3}$, and $9.5 \times 10^6$ U $m^{-3}$, respectively.

A cell line of photoautotrophic pak-bung hairy roots was established from photomixotrophic ones through acclimation cultures with a stepwise change of sucrose concentration in a medium with $CO_2$-enriched air supplied under continuous light irradiation. The derived photoautotrophic hairy roots had high Chl content and activity of RubisCO compared with the photomixotrophic and heterotrophic ones. Electron microscopic observation revealed that the photoautotrophic hairy root cells possessed well-developed chloroplasts. The activities of APx and GPx found in the hairy roots were comparable to those found in the leaves and roots of parent plants of pak-bung, respectively. The elongation rate of growing points of the hairy roots decreased with increasing DCMU concentration. The photoautotrophic hairy roots thus secured relatively high photosynthetic potentials and entirely depended on photosynthesis for obtaining carbon and energy sources.

Kinetic analyses of the growth and Chl formation were conducted in the hairy root cultures under light irradiation. Reduction in interval between branches was exhibited with increasing light intensity in both the cultures of photomixotrohic and photoautotrophic hairy roots. Furthermore, for photoautotrophic hairy roots, the elongation rate and decay rate of growing points also increased with increasing light intensity. Concerning Chl formation, the distribution of Chl content along the hairy roots was recognized in both the photoautotrophic and photomixotrophic hairy roots. A kinetic model was presented to formulate the growth and Chl formation of hairy roots in the cultures with light irradiation. The model could well express the growth profiles of photoautotrophic and photomixotrophic hairy root cultures, and Chl formation in the photomixotrophic hairy roots.

For clarification of the phenomenon observed when hairy root cells were subjected to the conditions where energy supplies to the cells were limited, maintenance energy for the cells was estimated by taking into account the sugar consumption rate and photosynthetic potential. In the photoautotrophic cultures of the hairy roots, no elongation was observed when the root lengths were less than $2.0 \times 10^{-2}$ m. Based on the Chl content, the carbon fixation rate of a single root with $2.0 \times 10^{-2}$ min length was estimated. The calculated value

was comparable to or somewhat lower than the maintenance energies for the hairy roots derived from the sugar consumption.

In this article it was indicated that the elongation rate of derived photoautotrophic hairy roots was suppressed depending on the concentration of DCMU, which is a kind of photosynthesis inhibitor applied as a herbicide, though heterotrophic growth of the hairy root was less sensitive to this agent. Based on this finding, the photoautotrophic hairy roots may be used to detect the presence of some herbicides in surroundings. Moreover, combined with the assays with the heterotrophic and photomixotrophic hairy roots, it might be also possible to evaluate various type herbicides with different working mechanisms.

## References


1. Pauls KP (1995) Biotech Adv 13:673
2. Yeoman MM, Yeoman CL (1996) New Phytol 134:553
3. Dörnenburg H, Knorr D (1995) Enzyme Microb Technol 17:674
4. DiCosmo F, Misawa M (1995) Biotech Adv 13:425
5. White FF, Sinkar VP (1987) In: Horn TH, Schell J (eds) Plant DNA infectious agents. Springer, Berlin Heidelberg New York, p 149
6. Toivonen L (1993) Biotechnol Prog 9:12
7. Flores HE, Curtis WR (1992) Ann N Y Acad Sci 665:188
8. Sauerwein M, Yamazaki T, Shimomura K (1991) Plant Cell Rep 9:579
9. Nabeshima S, Mano Y, Ohkawa H (1986) Symbiosis 2:11
10. Hilton MG, Rhodes MJC (1990) Appl Microbial Biotechnol 33:132
11. Toivonen L, Laakso S, Rosenqvist H (1992) Plant Cell Rep 11:390
12. Taya M, Yoyama A, Kondo O, Honda H, Kobayashi T (1989) Plant Tissue Culture Lett 6:159
13. Kino-oka M, Taya M, Tone S (1991) Kagaku Kogaku Ronbunshu 17:1012
14. Taya M, Sato H, Kino-oka M, Tone S (1994) J Ferment Bioeng 78:42
15. Nagatome H, Tsutsumi M, Kino-oka M, Taya M (2000) J Biosci Bioeng 89:151
16. Murashige T, Skoog F (1962) Physiol Plant 15:473
17. Porra RJ, Thompson WA, Kriedemann PE (1989) Biochim Biophys Acta 975:384
18. Asada K, Takahashi M, Nagata M (1974) Agric Biol Chem 38:471
19. Yamada Y, Kobayashi S, Watanabe K, Hayashi U, Yajima Y, Inoue H (1987) J Chem Tech Biotechnol 38:31
20. Somogyi M (1952) J Biol Chem 195:19
21. Horn ME, Sherrard JH, Widholm JM (1983) Plant Physiol 72:426
22. Racker E (1962) In: Colowick SP, Kaplan NO (eds) Methods in enzymology, vol 5. Academic Press, New York, p 266
23. Nakano Y, Asada K (1980) Plant Cell Physiol 22:867
24. Chen GX, Asada K (1990) J Biol Chem 265:2775
25. Kamisaka S, Larsen P (1997) Plant Cell Physiol 18:595
26. Spurr AR, Harris WM (1968) Amer J Bot 55:1210
27. Nishimura M (1987) Photosynthesis Iwanami Shoten, Tokyo, p 54
28. Asada K (1993) Nippon Nogeikagaku Kaishi 67:1255
29. Asada K, Kanematsu S, Takahashi M, Kona Y (1976) In: Yasunobu KT, Mower HF, Hayaishi O (eds) Iron and copper proteins. Plenum Press, New York London, p 551
30. Flores HE, Hoy MW, Pickard JJ (1987) Trends Biotechnol 5:64
31. Flores HE, Pickard JJ, Hoy MW (1988) In: Lam J, Breteler H, Arnason T (eds) Chemistry and biology of naturally occurring acetylenes and related compounds. Elsevier, Amsterdam, p 233

32. Yoshimatsu K, Satake M, Shimomura K (1990) J Nat Pro 53:1498
33. Aoki T, Toda Y, Shimomura K (1992) Plant Tissue Culture Lett 9:214
34. Flores HE, Dai Y, Cuello JL, Maldonado-Mendoza IE, Loyola-Vargas VM (1993) Plant Physiol 101:363
35. Groß U, Gilles F, Bender L, Berghöfer P, Neumann KH (1993) Plant Cell Tiss Org Cult 31:143
36. Pamplin EJ, Chapman JM (1975) J Exp Bot 26:212
37. Fischer U, Alfermann AW(1995) J Biotechnol 41:19
38. Rogers SMD, Ogren WL, Widholm JM (1987) Plant Physiol 84:1451
39. Groß EL (1980) In: San Pietro A (ed) Methods in enzymology, vol 69. Academic Press, New York, p 474
40. Sato F, Asada K, Yamada Y (1979) Plant Cell Physiol 20:193
41. Uozumi N, Makino S, Kobayashi T (1995) J Ferment Bioeng 80:362
42. Kino-oka M, Taya M, Tone S (1995) J Chem Eng Japan 28:772
43. Taya M, Kino-oka M, Tone S, Kobayashi T (1989) J Chem Eng Japan 22:698
44. Repunte VP, Kino-oka M, Taya M, Tone S (1993) J Ferment Bioeng 75:271
45. Nakashimada Y, Uozumi N, Kobayashi T (1994) J Ferment Bioeng 77:178
46. Repunte VP, Shimamura S, Taya M, Tone S (1994) J Chem Eng Japan 27:523
47. Hirayama S, Ueda R, Sugata K (1995) Energy Convers Mgmt 36:685
48. Hirata S, Taya M, Tone S (1998) J Chem Eng Japan 31:636
49. Hata J, Toyo-oka Y, Taya M, Tone S (1997) J Chem Eng Japan 30:315
50. Nagatome H, Yamamoto T, Taya M, Tanaka N (2000) Biochem Eng J 6:75

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# Author Index Volume 1–72

*Author Index Volumes 1–50 see Volume 50*

# Subject Index